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4

August 14, 2000

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET (Large Entity)

A/PROV

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

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☐ *Additional inventors are being named on page 2 attached hereto*

TITLE OF THE INVENTION (280 characters max)

WOUND HEALING FORMULATIONS CONTAINING HUMAN PLASMA FIBRONECTIN

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification — *Number of Pages* 135

☒ Drawing(s) — *Number of Sheets* 9,see p.2

☒ Other (specify) — claims -- 1 sheet

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☒ A check or money order is enclosed to cover the filing fees — FILING FEE AMOUNT

☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 15-0699 — $150.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE Manette Dennis DATE August 20, 1999

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P19LARGE/REV04

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INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (city and either State or Foreign Country)
Note: There are drawings interspersed with text in the specification. These drawings were not counted separately.		

60030 05/54 PTO

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WOUND HEALING FORMULATIONS CONTAINING HUMAN PLASMA FIBRONECTIN

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FIBRONECTIN SOLID WOUND DRESSINGS

Alternative methods of production

Composition of different solid wound dressings

Solid Carboxymethylcellulose (CMC) dressing

Solid carboxymethylcellulose (CMC) dressing was prepared. Preferred grade is GPR® (BDH Laboratories, Ville St-Laurent, Canada). A solid wound dressing containing (w/w) fibronectin 62%, CMC 38% was prepared as follows. CMC powder was first sterilized by using a dry-heat sterilization process. CMC (6g) was dispersed in 94 mL of deionized water and allowed to be mixed with a paddle type stirrer for about 3 hours. This provides a sterile concentrated hydrogel base (6% w/w). Lyophilized fibronectin (50 mg) was dissolved in deionized water (5 mL) containing 12 µL of NaOH 3M, pH 11.6. The solution was maintained at 37°C until complete solubilization of fibronectin occured. This stock solution of fibronectin 10 mg/mL was filtered through a 0.22 µm acetate filter. Fibronectin solution (3.3 mL) was then added to a portion (0.34 g) of concentrated CMC base and mixed into syringes. The pH is adjusted at 7.0 with the addition of 25 µL HCl 1 N. At this point, the homogenous solution of the fibronectin-CMC complex is deposited in a plastic mold and frozen. The water is then removed by freeze-drying. By this technique, a fibronectin-CMC wound dressing with a sponge-like structure is produced.

Solid Hydroxypropylcellulose (HPC) dressing

Solid hydroxypropylcellulose (HPC) dressing was prepared. Preferred grade is Klucel-HF® (Aqualon, Houston, Texas). A solid wound dressing containing (w/w) fibronectin 45%, HPC 55% was prepared as follows. HPC powder was first sterilized by using a dry-heat sterilization process. HPC (6 g) was dispersed in 94 mL of deionized water and allowed to be mixed with a paddle type stirrer for about 3 hours. This provides a sterile concentrated hydrogel base (6% w/w). Lyophilized fibronectin (50 mg) was dissolved in deionized water (5 mL) containing 12 µL of NaOH 3M, pH 11.6. The solution was maintained at 37°C

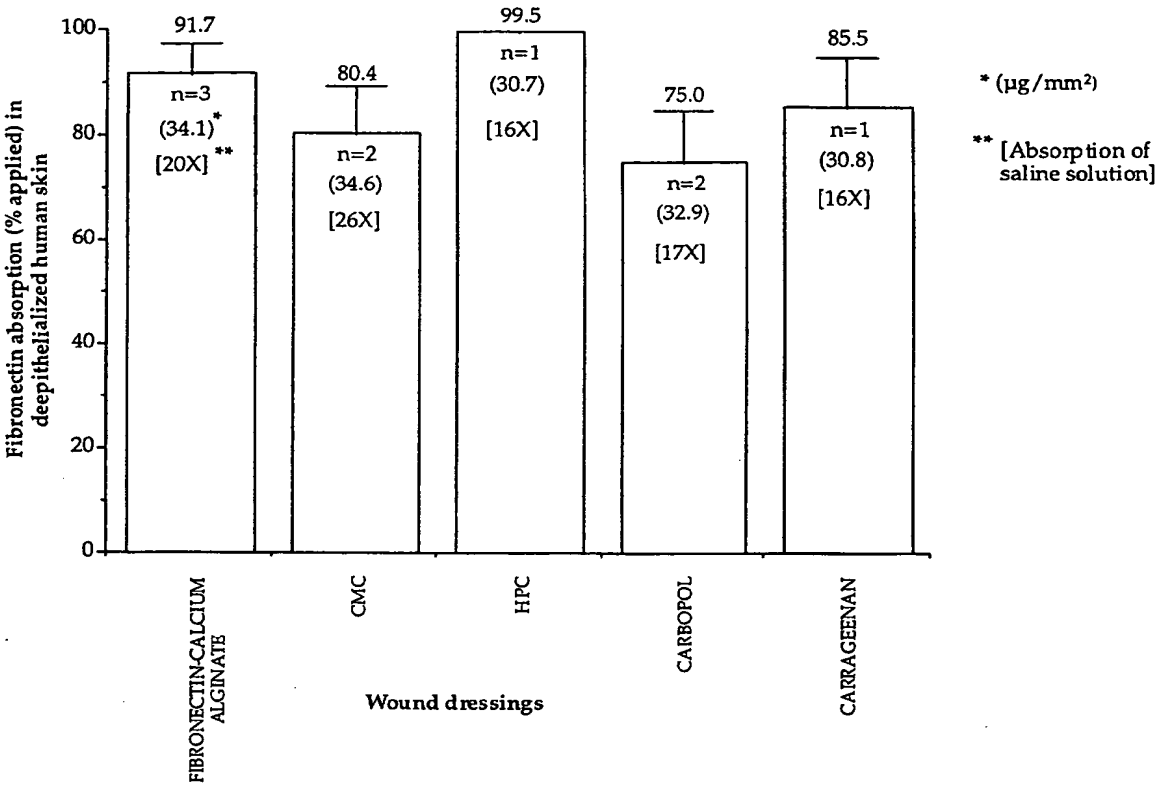
until complete solubilization of fibronectin occured. This stock solution of fibronectin 10 mg/mL was filtered through a 0.22 µm acetate filter. Fibronectin solution (3.3 mL) was then added to a portion (0.68 g) of concentrated HPC base and mixed into syringes. The pH is adjusted at 7.0 with the addition of 25 µL HCl 1 N. At this point, the homogenous solution of the fibronectin-HPC complex is deposited in a plastic mold and frozen. The water is then removed by freeze-drying. By this technique, a fibronectin-HPC wound dressing with a sponge-like structure is produced.

Solid carbomer dressing (Carbopol)

Solid carbomer dressing was prepared. Preferred grade is Carbopol® 974P NF (BF Goodrich, Cleveland, Ohio). A solid wound dressing containing (w/w) fibronectin 75%, carbomer 25% was prepared as follows. Carbomer (2.80 g) was dispersed in 97.2 mL of deionized water and allowed to be mixed with a paddle type stirrer for about 3 hours. This dispersion is then autoclaved to provide a sterile concentrated hydrogel base (2.80% w/w). Lyophilized fibronectin (50 mg) was dissolved in deionized water (5 mL) containing 12 µL of NaOH 3M, pH 11.6. The solution was maintained at 37°C until complete solubilization of fibronectin occured. This stock solution of fibronectin 10 mg/mL was filtered through a 0.22 µm acetate filter. Fibronectin solution (3.3 mL) was then added to a portion (0.04 g) of concentrated carbomer base and the necessary amount of gelifying promoter (25 µL NaOH 3M) and mixed with syringes. This fibronectin carbomer hydrogel is deposited in a plastic mold and frozen. The water is then removed by freeze-drying. By this technique, a fibronectin-carbomer wound dressing with a sponge-like structure is produced.

Solid carrageenan dressing

Solid carrageenan dressing was prepared. Preferred grade is Gelcarin® NF (FMC Corporation Pharmaceutical Division, Newark, Delaware). A solid wound dressing containing (w/w) fibronectin 73%, carbomer 27% was prepared as follows. Carrageenan (2.50 g) was dispersed in 97.5 mL of deionized water and allowed to be mixed with a paddle type stirrer for about 3 hours. This dispersion is then autoclaved to provide a sterile concentrated hydrogel base (2.50% w/w). Lyophilized fibronectin (50 mg) was dissolved in deionized water (5 mL) containing 12 µL of NaOH 3M, pH 11.6. The solution was maintained at 37°C until complete solubilization of fibronectin occured. This stock solution of fibronectin 10 mg/mL was filtered through a 0.22 µm acetate filter. Fibronectin solution (3.3 mL) was then added to a portion (0.50 g) of concentrated carrageenan base and mixed into syringes. The pH is adjusted at 7.0 with the addition of 60 µL HCl 1 N. At this point, the homogenous solution of the fibronectin-carrageenan complex is deposited in a plastic mold and frozen. The water is then removed by freeze-drying. By this technique, a fibronectin-carrageenan wound dressing with a sponge-like structure is produced.



Absorption of fibronectin in deepithelialized human skin using different solid wound dressings. The number in ( ) refers to the quantity of absorbed fibronectin (µg) per mm^2 of deepithelialized human skin over a 12 hour period. The number in [ ] refers to the quantity of absorbed saline solution (0.9% NaCl) by weight of dressing. Bars represent standard deviations of the mean.

Absorption of fibronectin in deepithelialized human skin using different solid wound dressings over a 24 hour period

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(54) ALGINATE WOUND DRESSINGS

ALGINATWUNDVERBÄNDE

PANSEMENTS A L'ALGINATE POUR BLESSURES

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(56) References cited:

EP-A- 0 476 756	WO-A-89/12471
WO-A-90/01954	WO-A-91/11205
WO-A-92/19802	WO-A-93/16111

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EP 0 721 355 B1

Description

The present invention relates to wound dressings, components thereof and to the methods of their manufacture. More specifically this invention relates to dressings suitable for use on exuding wounds, to alginate fibres for use therein and to the methods of their manufacture.

Exuding wounds such as ulcers, pressure sores and burns tend to produce copious volumes of exudate through at least part of the healing process. Many methods of coping with highly exuding wounds have been suggested, for example covering the wound with a highly permeable adhesive film, covering the wound with an adhesive hydrocolloid dressing or employing a dressing comprising an alginate. The use of an alginate wound dressing has proved to have many advantages, for example in terms of aiding healing of the wound as avoidance of damaging friable tissue on removal. Such dressings are described in: EP 89917135.6, EP 89910126.5 and EP 91202326.4.

Although known alginate dressings have good solubilities, there can be difficulties in removing them from wounds, for example in use they can partially dissolve to a weak gel which has to be washed from the wound. A more soluble dressing would be desirable to ease the removal by washing. Such a dressing has now been discovered.

In addition, it is known that alginate dressings have considerable disadvantages in that they, in common with other forms of dressing, do not sufficiently prevent tissues surrounding highly exuding wounds from becoming macerated. Maceration is not desirable because it can lead to increased friability of tissue. There is therefore a desire to provide a flat alginate dressing which is less likely to allow maceration of tissue surrounding highly exuding wounds. Such a flat dressing has now been discovered.

The present invention provides a wound dressing which comprises alginate characterised in that the alginate has a mannuronate content of 50% to 80%, has a molecular weight of 7000 to 40000 and has a monovalent : polyvalent ion content of from 10 to 30 : 70 to 90.

It has been found that dressings of the invention and the fibres of which they are composed hydrate rapidly. This greatly helps removal of the dressings and, in the case of flat dressings, significantly reduces the amount of lateral transport of fluid by capilarity. This in turn reduces the amount of exudate transported to the tissue surrounding the exuding wound so that the tendency for that tissue to become macerated is reduced or eliminated.

Alginates are produced by a variety of micro-organisms and marine algae which are the normal commercial source. The alginates being natural materials show considerable variety but are characterised in being block copolymers, the individual monosaccheride units being arranged into groups as blocks of mannuronic (M) and guluronic (G) residues. In addition to the repeating blocks each polymer chain can contain a proportion of alternating M and G monosaccharide units. It has been found that alginates containing 50% to 80% mannuronate can (if having an appropriate molecular weight and ionic content) be of particular use in wound dressings.

Alginates containing 50% to 85% of mannuronate can be obtained from species such as Ascophyllum nodosum, Durvillea Protatonum, Lasonia Nigrescens and Ecklonia Maxima. Blends may be used if desired. Favoured alginates for use in the dressings of this invention will contain from 60% to 80% and preferably from 70% to 75% of mannuronate. A suitable source of such materials is Kelco, Tadworth, Surrey, UK. Suitable grades include manacol of the appropriate molecular weight range.

It has been found that the desirable properties of the dressings are best achieved if the molecular weight of the alginate is from 5000 to 80000, aptly from 7000 to 40000, more suitably from 12000 to 35000 and preferably from 15000 to 30000, for example about 20000 to 25000. A fairly broad distribution of molecular weights within the alginate polymer population is acceptable. The molecular weight referred to is the number average molecular weight. One suitable method of determining the number average molecular weight is given in the descriptions hereinafter. (Thus the molecular weight is aptly at least 5000, more aptly at least 7000, yet more aptly at least 15000 and favourably, is at least 20000 aptly less than 80000, more aptly less than 40000, yet more aptly less than 35000 and more suitably less than 30000.

It has also been found that the desirable properties of the dressings are best achieved if the ratio between polyvalent ions (normally divalent ions) such as calcium and monovalent ions such as sodium is from 70 - 90 : 30 - 10, more suitably from 75 - 85 : 25 - 15 and preferably 80:20. Obviously small amounts of other ions may be present as long as they are pharmaceutically acceptable and do not interfere with the properties of the dressing.

A particularly suitable dressing of this invention will comprise alginate characterised in that the alginate has a mannuronate content of 60% to 80%, a molecular weight of 15000 to 25000 and a ratio of sodium ion to calcium ions of 15 to 25 : 75 to 85.

A preferred dressing of this invention will comprise alginate characterised in that the alginate has a mannuronate content of 70% to 75%, a molecular weight of about 20000 and a ratio of calcium ion to sodium ion 80:20.

The alginate is normally and preferably present as fibres. The fibres may be long or short, tangled or untangled, knitted or woven as desired. The dressing may be in the form of a flat needled or non-needled wound dressing, a sliver product (which is particularly suitable for cavities), a roving product (which is especially suitable for use in sinus cavities), an island dressing, as a yarn or knitted or woven fabric. The previously described patent applications (which are

incorporated herein by reference) may be inspected for suitable dressing [redacted] and manufacturing methods etc.

A wound dressing according to the present invention may further be provided with a moisture vapour permeable film, for example a polyurethane, polyetherester derivatives, a polyether amide and the like. Generally the polymers will be hydrophilic. Aptly such films will be from 15 to 50 microns thick, more usually 20 to 30 microns, for example 25 microns. The film layer may be applied directly to the surface of an alginate pad but more suitably will be adhered by means of an adhesive. Such adhesives are preferably moisture vapour permeable, for example an acrylic, polyurethane or polyether adhesive of which acrylic adhesives are preferred.

Dressings of this invention are normally provided sterile contained within a bacteria proof pouch.

A particularly suitable dressing of this invention comprises a pad of alginate fibres. These pads can be referred to as a "flat" dressing because they are generally produced flat but of course they can be flexible and adapt to the shape of a wound. In general these are provided as pads of from 4 x 4 x 0.1cm up to 20 x 20 x 0.3cm although other shapes and sizes are common. Dressings of this type are generally laid over exuding wounds such as ulcers or burns. The dressings of this invention do not need to be cut to shape to avoid maceration when used in this manner. They are particularly easy to remove because even if the wound covering portion gels or dissolves, the integrity of the wound surrounding portion is maintained and so allows the dressing to be lifted off. If any residual material is left in the wound it is particularly easy to remove by irrigation because of the high solubility of the fibres.

When used to cover exuding wounds and even when overwrapped with four layers of compression bandage, dressings of this invention were found not to cause maceration of surrounding skin and to be easily removed.

The dressings of this invention may be made in conventional manner, for example as described in the previously mentioned patents. They may contain antioxidants or preservatives if desired. The dressings may be sterilized by radiation if desired.

Description 1

A suitable method of molecular weight determination of alginate wound dressings.

Alginate solutions were prepared from wound dressing samples by titration with a concentrated aqueous Calgon solution (that is sodium hexametaphosphate, for example at 10% w/w). The Calgon solution was added dropwise to the wound dressing fibres, which were agitated with a magnetic stirring bar until the fibres had dissolved and a non-turbid solution was obtained. The concentrated (approximately 0.7%) alginate solutions were diluted with aqueous sodium nitrate to yield solutions containing approximately 0.2% alginate and 0.1M $NaNO_3$. The dilute alginate solutions were filtered through a 0.45 micron filter prior to injection into the Size Exclusion chromatography/Multiple Angle Laser Light Scattering instrument (SEC/MALLS).

Molecular weight distributions were determined by SEC/MALLS. Our suitable system comprises a Hewlett-Packard Liquid Chromatograph Model 1084B, a 30cm Ultrahydrogel Linear column (Waters) with a guard column, a DAWN Model F Light Scattering Detector and a Waters 410 Differential Refractometer. The sample is injected into the eluant flow (aqueous 0.1M $NaNO_3$ with 0.01% NaN_3 as an antimicrobial agent) and is separated based on molecular size by the size exclusion chromatography column. As the sample elutes from the column the molecular weight and concentration profiles are determined by the light scattering and refractive index detectors, respectively. An index of refraction increment (dn/dc) of 0.145 was used to determine the sample concentration (and molecular weight) as a function of elution volume. This value was obtained from the literature (Paoletti et al (1991) Carbohydrate Polymers, 15, 171; Mackie et al (1980) Biopolymers J-19, 1839; Strand et al (1982) Macromolecules, 15, 570) and is typical for alginates in aqueous salt solutions. The light-scattering detector was calibrated with a series of pullulan standards (Mw = 200,000; 400,000 and 800,000 g/mole) and a previously measured (by Paoletti) alginate sample of Mw = 210,000.

Weight-average and number-average molecular weights, as well as polydispersity indices (Mw/Mn), were determined for each sample. The molecular weight results for the algin samples are given in the table below.

Sample	Mn (g/mole)	Mw (g/mole)	Mw/Mn
Example 1	21,000	92,000	4.4
SORBSAN	120,000	300,000	2.5

The SORBSAN wound dressing has much higher and significantly different molecular weight distribution than the sample of Example 1 (Mw = 300,000 vs. 92,000 g/mole). Example 1 had a broader molecular weight distribution than that of SORBSAN.

Exa[redacted]

Manufacture of low molecular weight, high mannuronate, 80:20 alginate fabric

(a) Low molecular weight, high mannuronate calcium alginate fibre

The fibre was prepared by the method of preparation 1 of WO 90/01954 employing sodium alginate powder wherein the alginate had a number average weight molecular weight of 21,000 and had a mannuronate content of 70%.

(b) Low molecular weight, high mannuronate, 80:20 alginate fabric manufacture of

The fabric was prepared by the method of preparation 2 of WO 90/01954 but employing low molecular weight, high mannuronate calcium alginate fibre as prepared in part (a).

Examples 2-5

Alternative manufacture of low molecular weight, high mannuronate, 80:20 alginate fabric.

The fabrics were prepared by the methods of preparations 3 to 6 of WO 90/01954 adapted by the use of calcium alginate as prepared in Example 1(a) herein.

Example 6

A fabric as prepared in Example 1 was cut to 5cm x 5cm square and placed in the centre of a water vapour permeable polyurethane film of 8cm x 8cm square and 21 µm thick. The face of the film on which the fabric was placed was provided with a pressure sensitive adhesive layer comprising an acrylic adhesive. A silicone release paper was then placed on the remote face of the film and the paper was trimmed to size.

Claims

1. A wound dressing which comprises alginate characterised in that the alginate has a mannuronate content of 50% to 80%, has a molecular weight of 7000 to 40000 and has a monovalent : polyvalent ion content of from 10 to 30 : 70 to 90.

2. A dressing according to claim 1, which has a mannuronate content of 60 to 80%.

3. A dressing according to claim 2, which has a mannuronate content of 70 to 75%.

4. A dressing according to any of claims 1 to 3, wherein the alginate has a molecular weight of 12000 to 35000.

5. A dressing according to claim 4, wherein the alginate has a molecular weight of 15000 to 30000.

6. A dressing according to claims 1 to 5, which has a monovalent:polyvalent ion content of 15 to 25 : 75 to 85.

7. A dressing according to any of claims 1 to 6, wherein the monovalent ion is sodium and the polyvalent ion is calcium.

8. A wound dressing which comprises alginate characterised in that the alginate has a mannuronate content of 60% to 80%, a molecular weight of 15000 to 25000 and a ratio of sodium ion to calcium ion of 15 to 25 : 75 to 85.

9. A wound dressing which comprises alginate characterised in that the alginate has a mannuronate content of 70% to 75%, a molecular weight of about 20000 and a ratio of calcium ion to sodium ion 80:20.

10. A dressing according to any of claims 1 to 9, which further comprises a moisture vapour permeable film.

Patentansprüche

1. Wundverband, der ein Alginat umfaßt, dadurch gekennzeichnet, daß das Alginat einen Mannuronatgehalt von 50

80 %, ein Molekulargewicht von 7 000 bis 40 000 und einen von einwertigen : mehrwertigen Ionen von 10 bis 30 : 70 bis 90 aufweist.

2. Verband nach Anspruch 1, der einen Mannuronatgehalt von 60 % bis 80 % aufweist.

3. Verband nach Anspruch 2, der einen Mannuronatgehalt von 70 % bis 75 % aufweist.

4. Verband nach einem der Ansprüche 1 bis 3, wobei das Alginat ein Molekulargewicht von 12 000 bis 35 000 aufweist.

5. Verband nach Anspruch 4, wobei das Alginat ein Molekulargewicht von 15 000 bis 30 000 aufweist.

6. Verband nach einem der Ansprüche 1 bis 5, der einen Gehalt von einwertigen : mehrwertigen Ionen von 15 bis 25 : 75 bis 85 aufweist.

7. Verband nach einem der Ansprüche 1 bis 6, wobei das einwertige Ion Natrium und das mehrwertige Ion Calcium ist.

8. Wundverband, der ein Alginat umfaßt, dadurch gekennzeichnet, daß das Alginat einen Mannuronatgehalt von 60 % bis 80 %, ein Molekulargewicht von 15 000 bis 25 000 und ein Verhältnis von Natriumionen zu Calciumionen von 15 bis 25 : 75 bis 85 aufweist.

9. Wundverband, der ein Alginat umfaßt, dadurch gekennzeichnet, daß das Alginat einen Mannuronatgehalt von 70 % bis 75 %, ein Molekulargewicht von etwa 20 000 und ein Verhältnis von Calciumionen zu Natriumionen von 80:20 aufweist.

10. Verband nach einem der Ansprüche 1 bis 9, der ferner eine wasserdampfdurchlässige Folie umfaßt.

Revendications

1. Pansement pour blessures qui comprend un alginate, caractérisé en ce que l'alginate a une teneur en mannuronate de 50 % à 80 %, a un poids moléculaire de 7 000 à 40 000, et a une teneur en ions monovalents : polyvalents de 10 à 30 : 70 à 90.

2. Pansement selon la revendication 1, qui a une teneur en mannuronate de 60 à 80 %.

3. Pansement selon la revendication 2, qui une teneur en mannuronate de 70 à 75 %.

4. Pansement selon l'une quelconque des revendications 1 à 3, dans lequel l'alginate a un poids moléculaire de 12 000 à 35 000.

5. Pansement selon la revendication 4, dans lequel l'alginate a un poids moléculaire de 15 000 à 30 000.

6. Pansement selon l'une des revendications 1 à 5, qui a un teneur en ions monovalents : polyvalents de 15 à 25 : 75 à 85.

7. Pansement selon l'une des revendications 1 à 6, dans lequel l'ion monovalent est le sodium et l'ion polyvalent est le calcium.

8. Pansement pour blessures qui comprend un alginate, caractérisé en ce que l'alginate a une teneur en mannuronate de 60 à 80 %, un poids moléculaire de 15 000 à 25 000 et un rapport ion sodium à ion calcium de 15 à 25 : 75 à 85.

9. Pansement pour blessures qui comprend un alginate, caractérisé en ce que l'alginate a une teneur en mannuronate de 70 % à 75 %, un poids moléculaire d'environ 20 000 et un rapport ion calcium à ion sodium de 80 : 20.

10. Pansement selon l'une quelconque des revendications 1 à 9, qui comprend de plus un film perméable à la vapeur d'eau.

(even if the part covering the wound gels or dissolves, the part he wound retains its integrity and allows the dressing to be lifted off). Flat dressings show reduced lateral transport of exudate by capillarity, so reduce maceration of surrounding tissue, even when covered by a compression bandage. Dressings do not have to be cut to shad maceration. (Dwg.0/0)

?

For preparing 10 g of fibronectin carbomer hydrogel, the following ingredients must be added in sequence. First, the pH of 8.8 mL demineralized water pH 5.0 is adjusted at pH 8.0 to 11.0 with the addition of 2.95 µg to 2.95 mg NaOH 3M. The lyophilized fibronectin is next dissolved in demineralized water pH 8.0 to 11.0 in quantities varying from 0.05 to 0.1 g. In a final step of the procedure, 1 mL of water containing 0.028 g of carbopol and varying amount of NaOH 3M from 0.09399705 g to 0.09105 g are added to the mixture.

04.07.96

Essais de production de gels de Carbopol 0.375% avec 0.4% Fn (4mg Fn / g de gel)

- La protéine (Fn) purifiée est concentrée jusqu'à 2.2 mg/ml et lyophilisée.

① Gel à 0.4% Fn : 4mg Fn / g

Pour 5.0 g de gel : 0.5 g Carbopol 3.75%
4.4 ml H_2O
62.5 µl NaOH 3M
20 mg Fn

② Gel à 0.8% Fn : 8mg Fn / g

Pour 5.0 g de gel : 0.5 g Carbopol 3.75%
4.0 ml H_2O
62.5 µl NaOH 3M
40 mg Fn

Benoît Lasnier

15.08.96

Essais d'absorption cutanée de la Fn aux diverses concentrations dans un gel de Carbopol 0,375 %.

① Pour 3.0 g de gel : Gel à 0.4 % Fn : 4 mg Fn

0.2 g Carbopol 3.75 %
2.6 ml H_2O
37.5 µl NaOH 3M
12 mg Fn

② Pour 3.0 g de gel : Gel à 0.8 % Fn : 8 mg Fn/g

0.3 g Carbopol 3.75 %
2.6 ml H_2O
37.5 µl NaOH 3M
24 mg Fn

③ Pour 3.0 g de gel : Gel à 1.0 % Fn : 10 mg Fn/g

0.3 g Carbopol 3.75 %
2.6 ml H_2O
37.5 µl NaOH 3M
30 mg Fn

④ Contrôle :

2.9 mg/ml
- Par tube : 0.9 ml / 26 mg

05.08.96
10 ml 2.9 mg/ml

* SELF TEST *
COMPLETED

ID# 1 TEST02
CELL 1
595.0NM .796 A

ID# 2 TEST02
CELL 2
--- A

Benoît Lavigne

30.09.96

Production de gels à différentes concentrations en Carbopol par une concentration fixe de 1.0% Fn → ~~antibiotique~~ ; (40 µg NaOH / mg Carb

① Gel à 0.375% et 1.0% Fn (11.25 mg Carb.

Pour 3.0 g de gel : 0.3 g Carbopol 3.75%
2.67 ml H_2O
37.5 µl NaOH 3M (4.5 mg NaO
30 mg Fn

② Gel à ~~0.25~~ 0.225% et 1.0% Fn (6.44 mg Carbop

Pour 3.0 g de gel : 0.225 g Carbopol 3.75%
2.78 ml H_2O
28.1 µl NaOH 3M
30 mg Fn

③ Gel à 0.1875% et 1.0% Fn (5.62 mg Carbopol

Pour 3.0 g de gel : 0.15 g Carbopol 3.75%
2.82 ml H_2O
18.75 µl NaOH 3M
30 mg Fn

Fraunt Louisiane

12.03.97

Gel de fibronectine pour evaluer la stabilité à T° pièce

(1) Gel à 0.5% Fn et 0.28% Carbopol

20g de gel : 1.5g Carbopol 3.75%
18.2g H_2O
100mg Fn
187.5µl NaOH 3M

(2) Gel à 1.0% Fn et 0.28% Carbopol

20g de gel 1.5g Carbopol 3.75%
16.8g H_2O
200mg Fn
187.5µl NaOH 3M

→ 10g de gel conservés à 4°C
→ 10g de gel conservés à T° pièce

Tests à faire : électrophorèse Gel à 0.5%
Gelatin-binding
Adhésion cellulaire
Phagocytose en opsonisation

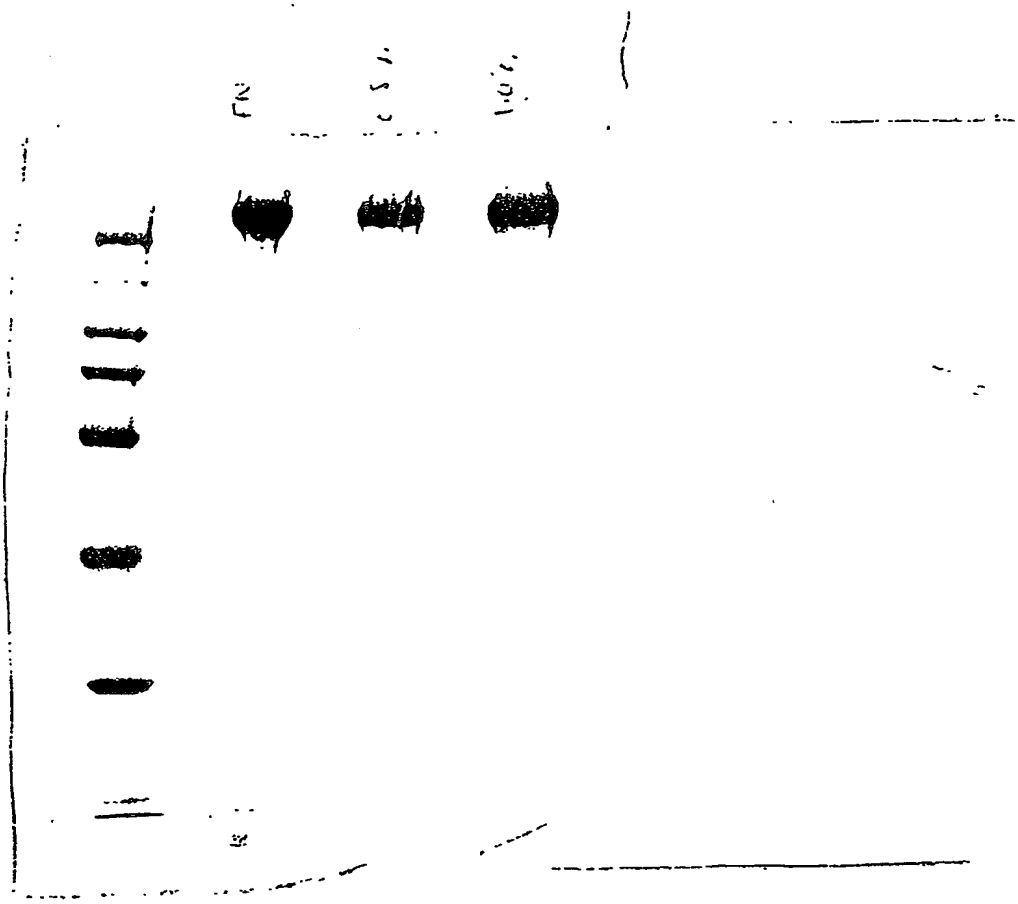
Temps. 0 - 3min - 6min - 9min - 1

Sont faites

- L'eau milli-Q est neutralisée avec NaOH ~~3M~~ 1N
  pH 9.0
- La FN est solubilisée à 37°C jusqu'à la disparition complète des agrégats avec l'eau milli-Q à pH 9.0. (10mg/ml)
- La solution de FN est par la suite filtrée sur 0,22.

13.03.97

Electrophorèse des gels à 0.5 % et 1.0 % de FN dans Carbopol 0.28 %.



Agent Lavinia 6500280 8665TOD

19.03.97

Test d'intégrité des gels 0.5 % et 1.0 % FN par la gélatine

voir page 1 (protocole)

20.03.97 IDEM

voir page 1 (protocole)

Composition of fibronectin carbomer hydrogels

Stock solution of carbomer			
3.75 %		2.80 %	
10 g (0.5% FN/ 0.28 % Carbomer)	10 g (1.0% FN/0.28 % Carbomer)	**10 g (0.5% FN/ 0.28 % Carbomer)**	**10 g (1.0% FN/0.28 % Carbomer)**
0.75 g Carbomer	0.75 g Carbomer	**1.00 g Carbomer**	**1.00 g Carbomer**
0.05 g FN	0.1 g FN	**0.05 g FN**	**0.1 g FN**
9.106 g water	9.056 g water	**8.856 g water**	**8.806 g water**
0.094 g NaOH 3M	0.094 g NaOH 3M	**0.094 g NaOH 3M**	**0.094 g NaOH 3M**

Stock solution of carbomer			
3.75 %		2.80 %	
20 g (0.5% FN/ 0.28 % Carbomer)	20 g (1.0% FN/0.28 % Carbomer)	**20 g (0.5% FN/ 0.28 % Carbomer)**	**20 g (1.0% FN/0.28 % Carbomer)**
1.50 g Carbomer	1.50 g Carbomer	**2.00 g Carbomer**	**2.00 g Carbomer**
0.1 g FN	0.2 g FN	**0.1 g FN**	**0.2 g FN**
18.212 g water	18.112 g water	**17.712 g water**	**17.612 g water**
0.188 g NaOH 3M	0.188 g NaOH 3 M	**0.188 g NaOH 3M**	**0.188 g NaOH 3M**

In order to prepare the fibronectin gel, the following ingredients must be added in this sequence. First, the pH of demineralized water (8.856 or 8.806 grams) is adjusted at 11.6 with 0.0235 grams of NaOH 3M. Lyophilized fibronectin is next dissolved in demineralized basic water (0.05 or 0.1 grams). In a final step of the procedure, 0.028 grams of carbomer and 0.0705 grams of NaOH 3M are added to the mixture. Please note the 0.028 grams of carbomer comes from 1.0 gram of carbomer stock solution of 2.8%. Please also note that the concentrated Carbomer start stock solution may be at 3.75% or 2.80%. This is illustrated in the right part of the table.

Solubility of fibronectin in non-buffered water (pH 5.0 - 6.0) compared to the solubility of fibronectin in water with NaOH 0.007 M, pH 11.6

Lyophilized fibronectin (mg)	Measured concentration of fibronectin (micro-Bradford method) (1 h /37°C in water) (mg/mL)	Measured concentration of fibronectin (micro-Bradford method) (12 h /37°C in water) (mg/mL)	Measured concentration of fibronectin by (micro-Bradford method) (24 h /37°C in water) (mg/mL)	Measured concentration of fibronectin by (micro-Bradford method) (1 h /37°C in water + NaOH) (mg/mL)
4	2.1 ± 0.4 (3)*	2.7 ± 0.4 (3)	3.0 ± 0.3 (4)	4.1 ± 0.2 (3)
10	5.7 ± 0.4 (3)	6.3 ± 0.2 (3)	5.7 ± 0.3 (4)	9.0 ± 0.3 (4)
15	7.1 (1)	7.5 (1)	--	--
20	7.3 (1)	8.5 (1)	--	14.1 (1)

*The number in parantheses refers to the number of experiments performed

Solubility of fibronectin in water containing varying concentrations of NaOH (10^{-7} M to 10^{-3} M) with corresponding pH (7.0 to 11.0)

Lyophilized fibronectin (mg)	Measured concentration of fibronectin (micro-Bradford method) (1 h /37°C in water + NaOH) (mg/mL)				
	10^{-7} M NaOH; pH 7.0	10^{-6} M NaOH; pH 8.0	10^{-5} M NaOH; pH 9.0	10^{-4} M NaOH; pH 10.0	10^{-3} M NaOH; pH 11.0
10	8.0 (1)*	8.5 (1)	9.2 ± 0.5 (2)	8.7 (1)	9.2 ± 0.3 (5)

*The number in parantheses refers to the number of experiments performed

US005877149A

United States Patent [19]

Beaulieu

[11] Patent Number: 5,877,149

[45] Date of Patent: Mar. 2, 1999

[54] **DEEPITHELIALIZED SKIN DIFFUSION CELL SYSTEM**

[76] Inventor: **André Beaulieu**, 4045, Chemin St.-Louis, Cap-Rouge (Québec) G1Y 1V7, Canada

[21] Appl. No.: **879,159**

[22] Filed: **Jun. 19, 1997**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 488,253, Jun. 7, 1995, Pat. No. 5,641,483.

[51] **Int. Cl.**[6] **G01N 21/00**; G01N 21/75

[52] **U.S. Cl.** **514/8**; 436/63; 436/87; 436/164; 436/166; 436/175; 436/804; 436/805; 436/807; 436/825

[58] **Field of Search** 436/63, 87, 164, 436/166, 175, 804, 805, 807, 825; 514/8

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,594,884	6/1986	Bondi et al.	73/64.3
5,457,093	10/1995	Cini et al.	514/12

Primary Examiner—Frederick Krass

Attorney, Agent, or Firm—Manette Dennis; Ostrager, Chong, Flaherty & Onofrio, P.C.

[57] **ABSTRACT**

A deepithelialized skin cell diffusion system which can be used to select topical gel and cream formulations containing human other wound healing promoters such as plasma fibronectin. The formulations provide slow release and increased contact time of fibronectin or other wound healing promoters to the wound site leading to its effective absorption.

9 Claims, 7 Drawing Sheets

(1 of 7 Drawings Sheet(s) Filed in Color)

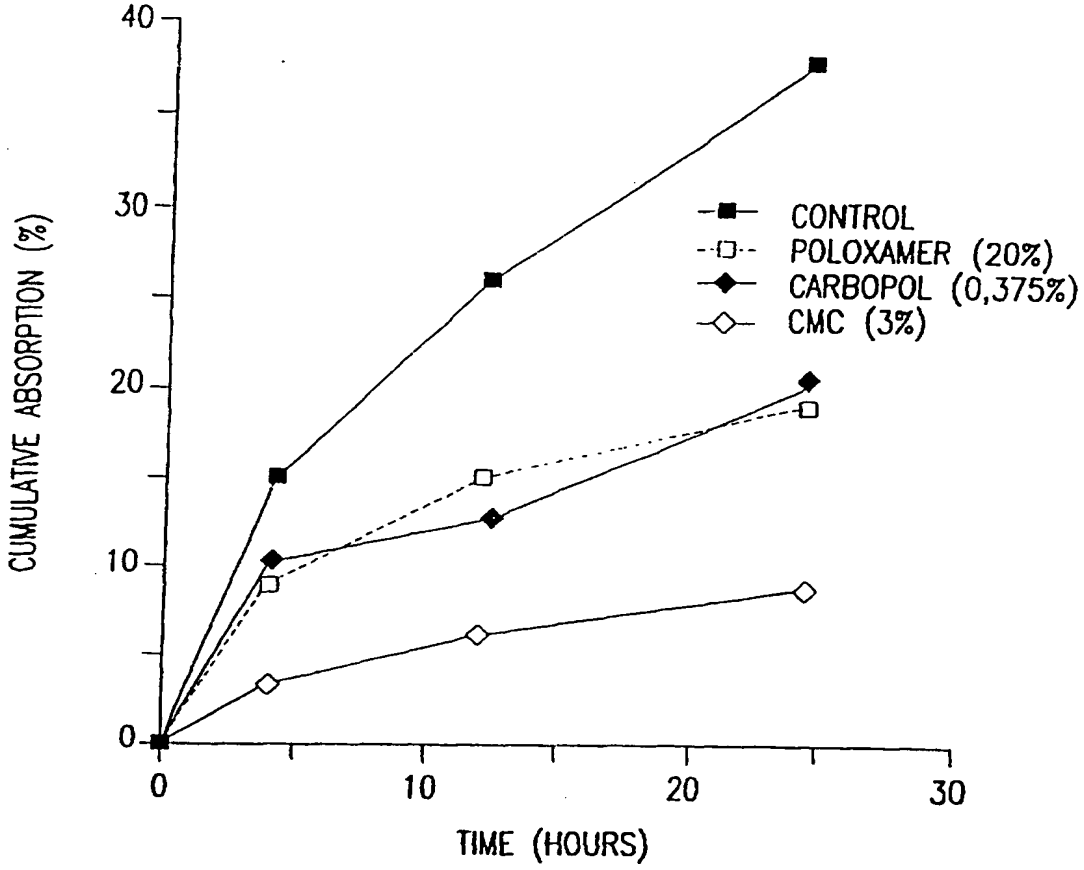

40
30
20
10
0
CUMULATIVE ABSORPTION (%)
0
10
20
30
TIME (HOURS)
CONTROL
POLOXAMER (20%)
CARBOPOL (0,375%)
CMC (3%)

FIG. 1

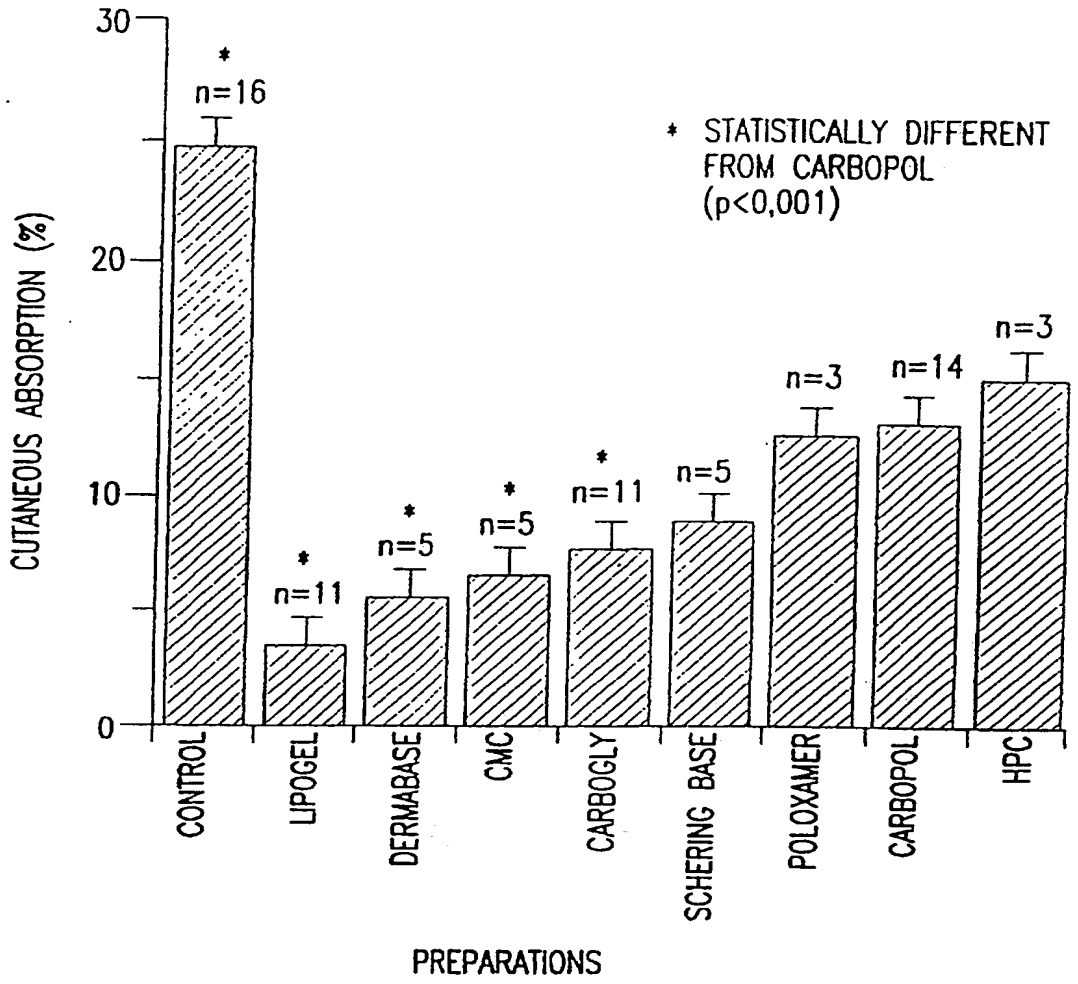
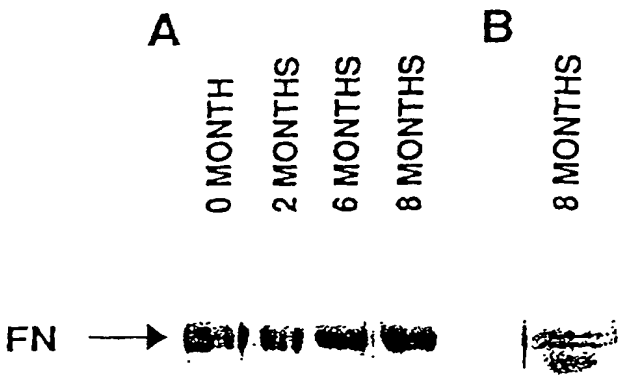

30
20
10
0
CUTANEOUS ABSORPTION (%)
* STATISTICALLY DIFFERENT FROM CARBOPOL (p<0,001)
*
n=16
*
n=11
*
n=5
*
n=5
*
n=11
n=5
n=3
n=14
n=3
CONTROL
LIPOGEL
DERMABASE
CMC
CARBOGLY
SCHERING BASE
POLOXAMER
CARBOPOL
HPC
PREPARATIONS

FIG.2

Figure 3

Stability of fibronectin in gel



Electrophoresis of human plasma fibronectin (FN) incorporated in a Carbopol P-934 gel after 0, 2, 6, and 8 months. Section A: Recovery of FN after a gelatin-binding test. Section B: Integrity of FN after 8 months in gel. It should be noted that in section B, the resolution of the band is affected by the presence of contaminants such as Carbopol in the specimen.

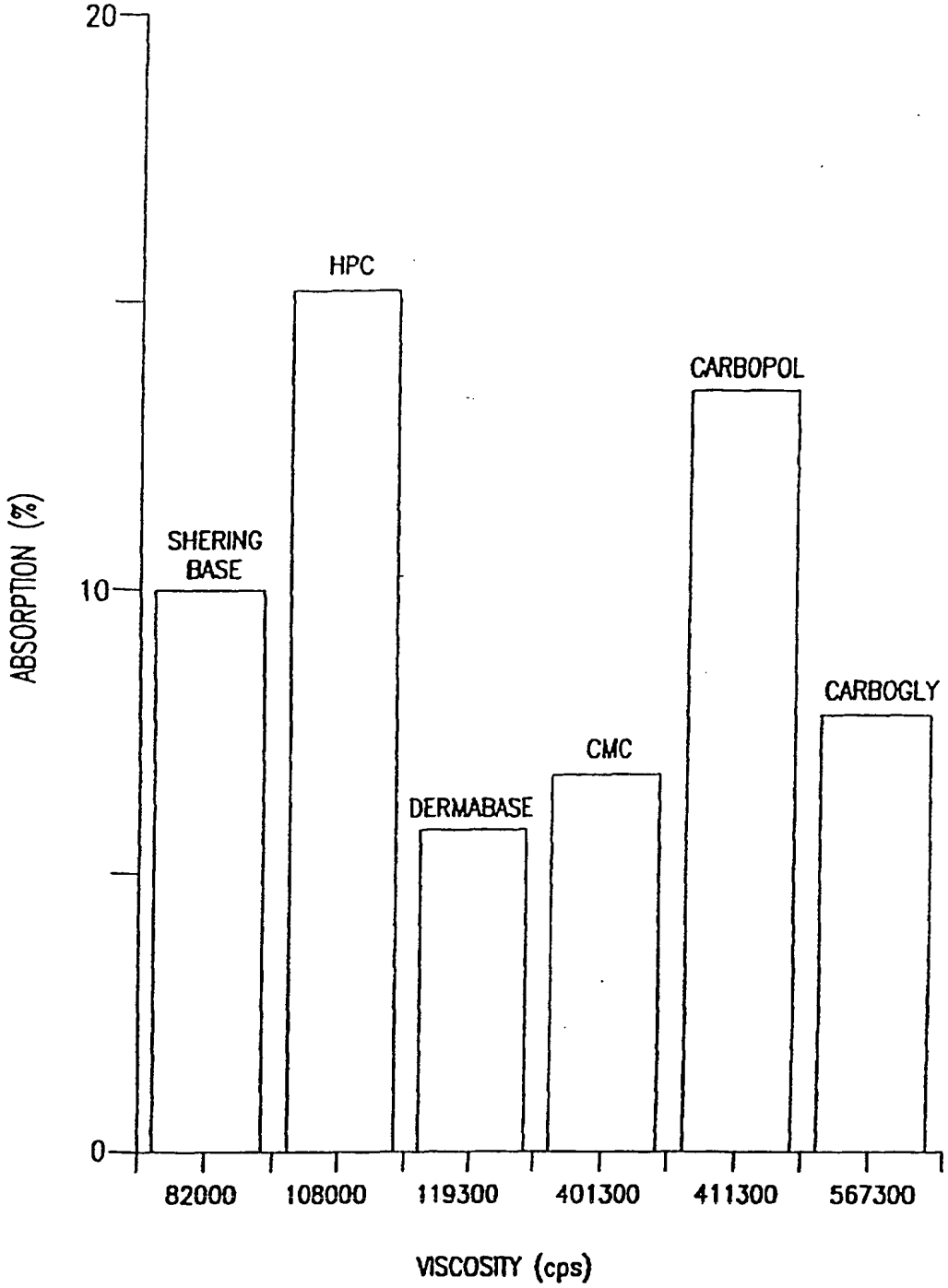

20
10
0
ABSORPTION (%)
SHERING BASE
HPC
DERMABASE
CMC
CARBOPOL
CARBOGLY
82000
108000
119300
401300
411300
567300
VISCOSITY (cps)

FIG.4

Fig 5: Absorption values in deepithelialized skin using increasing concentrations of FN in carbomer gel containing 0,28% of carbomer.

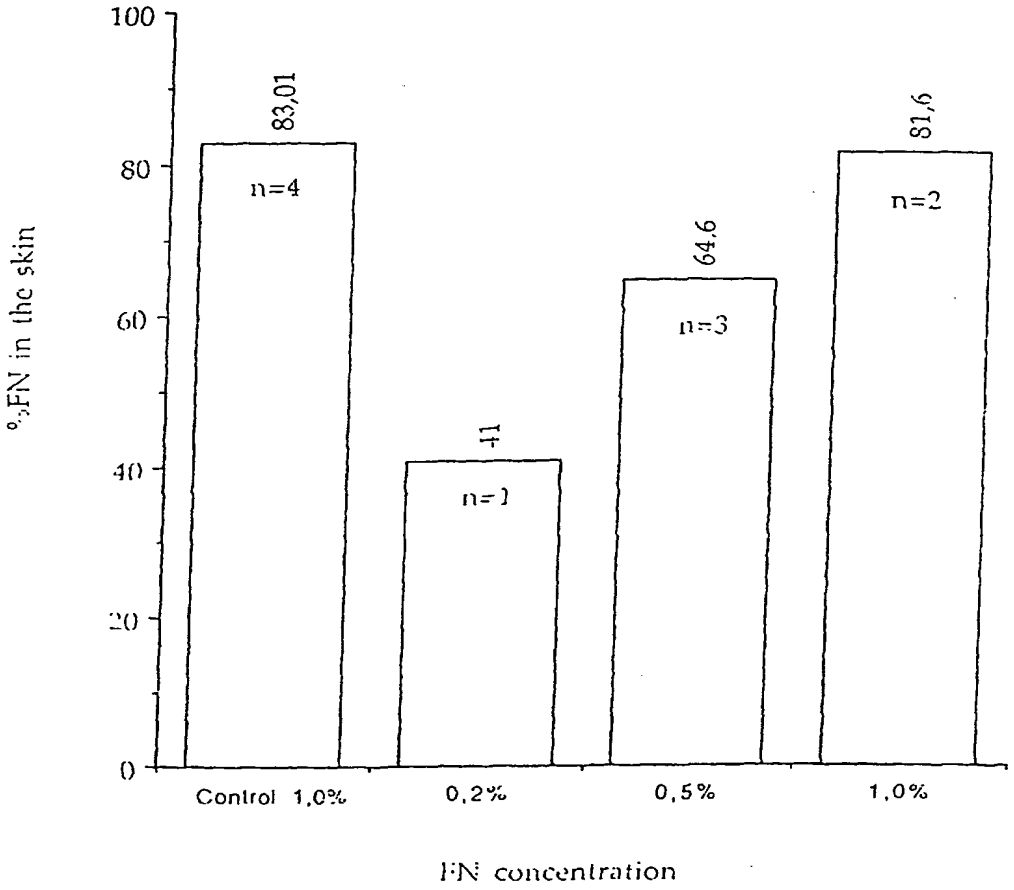


Fig 6: Effect of two different concentrations of carbomer on absorption values of FN in deepithelialized skin.

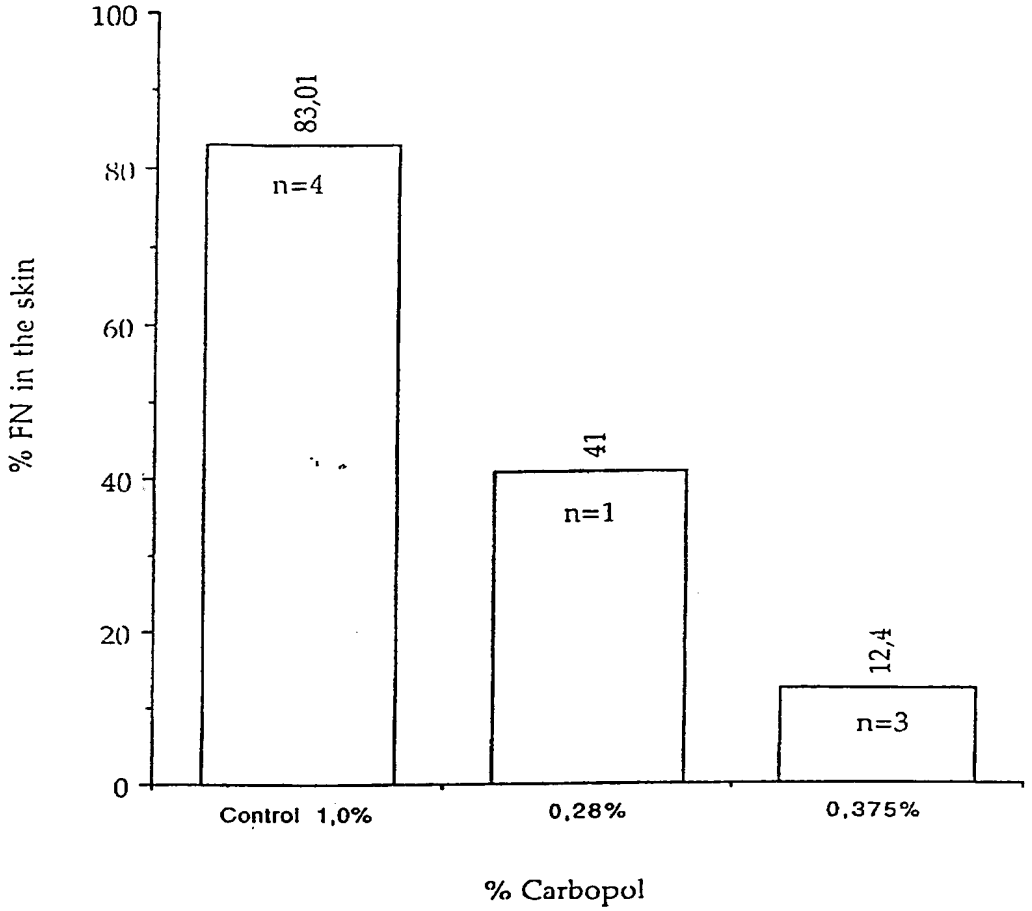


FIG. 7A

FIG. 7B

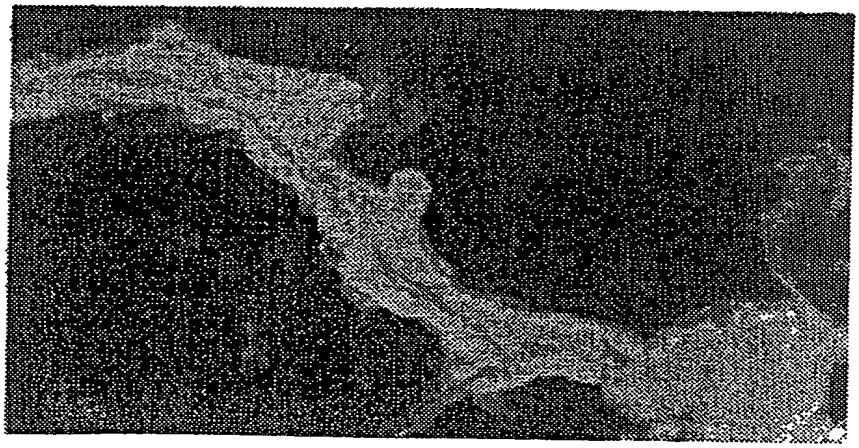


FIG. 7C

DEEPITHELIALIZED SKIN DIFFUSION CELL SYSTEM

RELATED APPLICATION

This application is a continuation-in-part of U.S. application Ser. No. 08/488,253, filed Jun. 7, 1995 now U.S. Pat. No. 5,641,483 for Wound Healing Formulations Containing Human Plasma Fibronectin. This application is a continuation-in-part of U.S. application Ser. No. 08/767,868, filed Dec. 17, 1996, also entitled: "Wound Healing Formulations Containing Human Plasma Fibronectin."

FIELD OF THE INVENTION

The present invention relates to topical dosage formulations, containing human plasma fibronectin and other wound healing promoters, for use in promoting wound healing in humans. In particular, the invention relates to the healing of chronic venous ulcers. A deepithelialized skin cell diffusion system is described which can be used to select suitable topical dosage formulations.

BACKGROUND OF THE INVENTION

Fibronectin is a large glycoprotein containing around 5% carbohydrate. The characteristic form of plasma fibronectin is a disulfide-bonded dimer of 440,000 daltons, each subunit having a molecular weight of about 220,000 daltons. Normally found in plasma at a concentration of about 300 μg/ml, fibronectin is extracted and purified using a method described by Hynes[1]. Plasma fibronectin is also known by various other names, including cold-insoluble globulin, antigelatin factor, cell attachment protein, cell spreading factor, and opsonic α2-surface binding glycoprotein. These names reflect biological activities of fibronectin such as cell recruitment, opsonization of particulate debris, and promotion of wound contraction. Reviews on structure and activities of fibronectin have been published elsewhere[2,3].

Wound healing is usually divided into three phases: the inflammatory phase, the proliferative phase, and the remodeling phase. Fibronectin has been reported to be involved in each stage of the wound healing process, particularly by creating a scaffold to which the invading cells can adhere. Initially, many mediators, such as fibronectin and fibrinogen, are released to the wound site. Fibronectin promotes inflammatory cells migration into the wound and debris phagocytosis by the monocytes. Thereafter, angiogenesis and reepithelialization take place. At this stage fibronectin exerts chemotactic activity on endothelial cells, and promotes the migration of epithelial cells and fibroblasts onto the basal membrane. Fibronectin also appears to be an essential component of the remodeling phase where it plays a major role in the organization of collagen fibrils. The fibrillar collagen ultimately forms fibrous bundles that greatly enhance the tissue tensile strength, leading to wound closure.

Topically applied plasma fibronectin has been reported as being useful for increasing the rate of wound healing such as in corneal wounds[4,5] and leg ulcers[6]. However, no one has described a suitable topical carrier for use in treating wounds that can ensure the delivery of an effective amount of fibronectin. A major limiting factor in developing an effective topical dosage form of a drug is not only having an active drug, but also having a formulation that allows the passage of the active drug from the carrier (cream, ointment, gel, etc.) into the site of delivery (which in the case of the present invention is a skin wound). Very active drugs, such as growth factors, may have no therapeutic value if the topical formulation does not allow the drug to move from the semi-solid carrier into the wound. Therefore, it would be highly desirable to develop a formulation which would maximize the contact time of the fibronectin with the wound and also control the release of fibronectin to the wound, thereby leading to high absorption values. The present invention provides such delivery system in the form of aqueous gels and a cream.

SUMMARY OF THE INVENTION

The present invention provides aqueous gel formulations and one cream formulation containing fibronectin and their use for the delivery of an effective wound healing amount of fibronectin to a wound site. The gel formulation comprises a water soluble, pharmaceutically acceptable polymer which is prepared from an effective amount of fibronectin. Examples of such compounds include: vinyl polymers, e.g. polyacrylic acid; polyoxyethylene-polyoxypropylene block copolymers, e.g. poloxamer; and cellulose derivatives, e.g. hydroxypropylcellulose (HPC). The polymer provides viscosity values between 50,000 and 1,000,000 cps at room temperature. The cream formulation is prepared from a commercially available cream base i.e., Schering® base (Schering Canada Inc., Point-Claire, Quebec), which has viscosity values between 60,000 to 80,000 cps at room temperature.

Many advantages are attributed to these dosage forms. Gel and cream formulations of the present invention release effective amounts of a wound healing promoter. Other advantages of gel formulations include: ability to keep the wound moist (which results from the high water content of the gels), ease of application and removal (by washing) from the wound. They also provide a cool feeling when topically applied which can increase patient comfort.

The slow release system of gel formulations of the present invention provides extended release of fibronectin to the wound site. This property of these formulations permits less frequent application to the wound resulting in less disturbance to the healing process. Such formulations maintain fibronectin delivery for up to 24 hours; but according to kinetic data obtained from permeation studies, a "twice a day" therapeutic schedule is a preferred embodiment of the present invention.

Formulation of topical dosage forms intended for the incorporation of fibronectin should respect several quality criteria. All components of the preparation including solvent, gelling agent and preservative should be nontoxic for the wound and compatible with the drug. The final product should promote optimal release of the drug to its site of action, be of adequate consistency to enhance contact time of the drug with the wound and be sterile.

The preferred formulations of this invention can be used with other wound healing promoters having a composition similar to fibronectin, such as proteins of similar size (thrombospondin, laminin, vitronectin, fibrinogen) or smaller size (such as peptides including growth factors).

The preferred formulations can be correlated with the results of evaluating the formulations using an in vitro diffusion cells system consisting of a rigid receptor containing a deepithelialized skin sample, the deepithelialized side facing upwards into a donor compartment and the dermal side facing downwards into a receptor compartment. The receptor compartment is connected to a circulating buffer circuit, with the buffer temperature maintained at 37° C. while the skin surface is at about 32° C. Preferred compositions will have an Abs value of greater than 7.8 preferably at least 13.40.

In addition to evaluating formulations for wound healing, the deepithelialized skin diffusion cell system could be used to evaluate the efficacy of other dermatological formulations such as analgesics, corticosteroids and antibiotics.

Furthermore, the dermal absorption of potentially toxic compounds which are suspended in various topical formulations can be avoided. For example, the deepithelialized skin diffusion cell system could be used to select formulations containing an insecticide which impede the absorption of the insecticide by broken skin. Other examples would include sun-screens and cosmetics.

Deepithelialized skin diffusion cells systems using deepithelialized skin samples from non-human animals can be used to test formulations for veterinary applications.

A preferred method of preparing the gels of the invention is to concentrate human fibronectin in demineralized water, which contains the polymerization promoter (NaOH). For higher concentrated fibronectin gels (0.5–1.0%), it is preferable to lyophilize the fibronectin. In both cases, the resulting solutions have a pH of about 9.0 to about 11.0. In this manner, it is possible to produce highly concentrated, non-precipitating solutions of fibronectin without using buffers, such as saccharides or stabilizers (e.g. albumin). Concentrated solutions of fibronectin, 2mg/ml to 10 mg/ml, can be reliably achieved using these methods.

The appropriate concentrated fibronectin solution is mixed with a concentrated solution of gelling agent. The two solutions are mixed by multiple exchanges under pressure using devices, such as syringes, which do not vigorously agitate the mixture in order to avoid fibronectin precipitation. The mixing devices are connected by an adaptor device.

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing (s) will be provided by the Patent and Trademark Office upon request and payment of necessary fee.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 depicts the cumulative absorption of radiolabelled fibronectin over time from various gel formulations containing Carbopol® P-934 carbomer (0.375%), Pluronic® F127 poloxamer (20.0%), sodium carboxymethylcellulose (CMC 3.0%), and from the control (phosphate buffered saline solution).

FIG. 2 depicts cutaneous absorption of radiolabelled fibronectin from various dosage forms and from the control (phosphate buffered saline solution) at time=12 hours.

FIG. 3 depicts the electrophoresis of human plasma fibronectin (FN) incorporated in a Carbopol® carbomer gel (Carbopol® P-934 carbomer 0.375%+chlorocresol 0.1%) after 0, 2, 6, and 8 months. Section A: Recovery of FN after a gelatin-binding test. Section B: Integrity of FN after 240 days of storage in gel at 4° C. It should be noted that in section B, the resolution of the band is affected by the presence of contaminants such as Carbopol® carbomer in the specimen.

FIG. 4 shows a plot of dermal absorption versus viscosity from different topical preparations.

FIG. 5 demonstrates the absorption values in deepithelialized skin using increasing concentrations of fibronectin in carbomer gel containing 0.28% carbomer and from the control (phosphate buffered saline solution).

FIG. 6 illustrates the effect of two different concentrations of carbomer on absorption values of fibronectin in deepithelialized skin. Both carbomer formulations contain 0.2% fibronectin; the control is phosphate buffer solution.

FIG. 7 depicts the immunofluorescence of fibronectin which can be detected in 2 μm thick frozen deepithelialized skin section after an absorption time of 12 hours in a diffusion cell system –7A. Negative control; Carbopol® carbomer gel containing no fibronectin, at 8 μm depth 7B. 0.5% (w/w) fibronectin in Carbopol® carbomer gel at around 482 μm skin depth; 7C. 0.5% (w/w) fibronectin in Carbopol® carbomer gel at around 2000 μm skin depth.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides dosage forms that are specially formulated for the therapeutic use of fibronectin as a topical wound healing promoter. The dosage forms selected for topical applications should ideally release large amounts of fibronectin, be sterile and non-toxic for the wound. Several factors such as physico-chemical properties of the glycoprotein as well as clinical utilization criteria must be considered when compounding these formulations.

Among these limitations, the major one relates to the solubility of fibronectin in water which is poor and therefore mitigates against the preparation of concentrated gels or creams. Fibronectin is only slightly soluble in water and may precipitate at concentrations as low as 5 mg/ml in aqueous solution. Its solubility is also affected by pH changes and low temperatures. In the same way, formulations that require the dispersion of polymer powder in the fibronectin solution under agitation cannot be prepared easily since precipitation of the glycoprotein may occur. Under agitation, fibronectin may aggregate and form long mats of insoluble material. Viscosity must be optimal in order to permit a sufficient adherence to the wound as well as good release capabilities.

Temperatures over 60° C., which are frequently required to provide sterile preparations, denature fibronectin. Since a terminal sterilization process cannot be performed on the final product, the preparation of concentrated bases of vehicles without fibronectin is usually unavoidable. Portions of these sterile bases are then diluted with a definite amount of a solution of fibronectin. To achieve adequate dispersion of fibronectin into semi-solid dosage forms, an incorporation step involving agitation is often required which can lead to the precipitation of the drug.

Gelling agents such as Carbopol® carbomer and poloxamer can circumvent this problem since they are sterilized before gelation under a liquid-like, viscous form. A highly concentrated preparation of Carbopol® carbomer is prepared and autoclaved. As described below, the solution of fibronectin which also contains the polymerization promoter (NaOH) is then mixed in syringes with the base of Carbopol® carbomer, building-up the gel during the dispersion of the drug into it. In the case of poloxamer, the polymer is added to the drug solution and allowed to dissolve at 4° C., a temperature at which it maintains its fluid-like aspect. Sterilization of this solution from bacteria is performed at 4° C. using a 0.22 μm filter.

A non-toxic, non-sensitizing preservative compatible with formulation components is added to the dosage form in a preferred embodiment of the invention. All of the above conditions are respected in the preferred dosage forms described in detail as follows.

An effective wound healing amount of human plasma fibronectin for use in the present invention is within the range of about 0.05 to about 1.0%, preferably about 1.0%. Fibronectin is isolated from human plasma by using a gelatin-Sepharose affinity chromatography procedure. In this method, gelatin is covalently coupled to Sepharose 4B

after CNBr activation. The binding capacity for human plasma fibronectin provided by this system is >1 mg/ml of gel.

Autologous, homologous human plasma fibronectin or fibronectin obtained from recombinant-DNA technology may be used in the present invention[1-7]. Should homologous plasma fibronectin be used, lots prepared from different donors would have to be tested for atypical antibodies, hepatitis B (HBV), hepatitis C (HCV), human immunodeficiency virus (HIV), human T-cell lymphotrophic virus (HTLV), cytomegalovirus (CMV) and syphilis. These tests have to be performed on donors just prior to the donation and 6 months thereafter. In the meantime, donor plasma must be kept frozen at −20° C. Furthermore, special steps should be taken in order to inactivate potential viruses. An inactivation method using tri (n-butyl) phosphate/Tween-80 or tri (n-butyl) phosphate/Triton X-100 (solvent/detergent method) should be performed on all plasma donations[7,8].

In the gel formulation for topical wound healing, the viscosity may be within the range of 50.000 to 1.000.000 cps, more preferably between 100.000 and 650.000 cps. In the cream formulation, the viscosity may be within the range of 60.000 to 80.000 cps. All viscosity values are in centipoises (cps) as measured using a Brookfield viscometer. Assays were performed at 0.5 rpm and at room temperature.

In one embodiment of the present invention, the gel formulation may comprise 0.25 to 1.0% by weight polyacrylic acid having a molecular weight of about 740.000 to 5.000.000. In a preferred embodiment, the polyacrylic acid is present at 0.35 to 0.75% by weight and has a viscosity of about 350.000 cps. The pH of the polyacrylic acid gel should be within the range of 5 to 8 and more preferably between 6.5 and 7.5. Polyacrylic acid, also known as carbomer, is sold under the trademark Carbopol® (BF Goodrich Company, Specialty Polymers and Chemical Division, Cleveland, Ohio). The preferred grade of Carbopol® carbomer is P-934.

In another embodiment, the gel formulation may comprise 18 to 35% by weight polyoxyethylene-polyoxypropylene block copolymer having a molecular weight of about 2,000 to 13,000. In a preferred embodiment, the polyoxyethylene-polyoxypropylene block copolymer is present at 18 to 25% by weight and has a viscosity of about 450,000 cps at room temperature. The pH of the block copolymer gel should be within the range of 6 to 8 and more preferably between 6.5 and 7.5. Polyoxyethylene-polyoxypropylene block copolymers, also known as poloxamer, is sold under the trademark Pluronic® (BASF Company, Mount Olive, N.J.). The preferred grade of Pluronic® poloxamer is F-127 (poloxamer 407).

In a further embodiment, the gel formulation may comprise 1 to 5% cellulose derivative which may be hydroxypropylcellulose (HPC) and has a viscosity of about 25.000 to 150.000 cps. HPC has a molecular weight of about 370.000 to 1,150.000. In a preferred embodiment, the cellulose derivative is present at 2.0 to 4.0% by weight and has a viscosity of about 150.000 cps for HPC. Cellulose derivatives used in the present invention are commonly known as Klucel HPC (Hercules Incorporated, Wilmington, Del.). Preferred grade is Klucel-HF.

In a further embodiment, a cream formulation is prepared from a commercially available cream base i.e., Schering® base. This cream base (oil in water emulsion) contains ceteth-20, cetostearyl alcohol, chlorocresol, mineral oil, monobasic sodium phosphate, phosphoric acid, sodium hydroxide, water and white petrolatum. The viscosity of the preparation can be modified by varying the content of water and polyethylene glycol.

Formulations of the present invention contain an aqueous phase in combination with a protein and thus are prone to attack by bacteria and fungi. Microbial growth not only spoils the formulation but is a potential toxicity hazard and a source of infection for patients. Even though microbial growth is less likely to be dangerous when it occurs in a topical preparation, it is especially important to preserve topicals which patients have to apply to broken or inflamed skin. Viscosity degradation reported with some polymers when exposed to microbial contamination is also of concern. So, a preservative should be added to the preparation to guarantee long term sterility and stability. The present invention provides gels that comprise a preservative selected from phenol or the para-hydroxybenzoate compounds. In one embodiment, the gel formulation may contain 0.1 to 0.2% by weight chlorocresol, a phenol derivative or 0.01 to 0.3% by weight p-hydroxybenzoate as methyl- and propylparaben. In another embodiment, the cream formulation contains 0.1 to 0.2% by weight chlorocresol.

Stabilizers may be added to the formulation in order to provide stable compositions of fibronectin. They may help to preserve biological activities on a long term basis and may improve water solubility of fibronectin. Among these agents, albumin, disaccharides such as sucrose, and cyclic oligosaccharides such as cyclodextrins are stabilizers of choice. These agents can be used either alone or in combination. Human albumin is preferable in terms of antigenicity and should be free from microbial contamination. Cyclodextrins of the β group (7 glucose units) are of choice and hydroxypropyl-β-cyclodextrin is preferable. The formulation may comprise 0.01 to 0.1% by weight albumin, preferably 0.01 to 0.05%; and/or 0.5 to 5.0% by weight sucrose, preferably 3.0 to 5.0%; and/or 1.0 to 10% by weight hydroxypropyl-β-cyclodextrin, preferably 2.0 to 5.0%.

Some authors have suggested that protease activity in some chronic wounds may cause degradation of adhesion proteins such as fibronectin and prevent cell adhesion necessary for normal wound closure[9]. Metalloproteases and serine proteases have been identified in chronic wound fluid[9,10] and fibronectin has been reported to be highly sensitive to cleavage by proteases[11]. Protection of the integrity of fibronectin may be accomplished by the addition of protease inhibitors in the dosage form. The present invention also provides formulations that may comprise a metalloprotease inhibitor such as EDTA and/or a serine protease inhibitor such as aprotinin (Trasylol®, Miles) with this aim in view. In one embodiment, the dosage form may comprise 0.01 to 1.0% by weight EDTA and/or 1.5 to 45.0 Inh U % by weight aprotinin where 1 Inh U=26 Kallikrein inhibitor units.

Formulations of the present invention can be applied to the wound site by any suitable means which assures that the wound surface will be entirely covered. For example, it can be directly applied to the wound site or used to coat fibers of an absorbent gauze dressing to form a wound healing bandage which may then be placed on a wound.

Examples which follow are intended to illustrate further aspects of the invention and are not to be construed as limiting its scope in any way.

EXAMPLE 1

Isolation of fibronectin from human plasma

1) A sterilization step is mandatory for all homologous plasma donations. In order to inactivate potential viruses, a sterilization procedure using the s/solvent/detergent method

is used. 1% tri (n-butyl) phosphate (TNBP) and lo Triton X-100 are added to the plasma for 6 hours at 24° C. After that, soybean oil is added to the plasma and allowed to be mixed for at least 30 minutes in order to extract TNBP. Residual Triton will be eliminated by dialysis.

This first step is skimped if autologous plasma is used.

2) A gelatin-Sepharose 4B column is first prewashed with a Tris-HCl solution in order to equilibrate the gel.

3) The plasma is diluted (1:1) with a Tris-Hcl solution and pumped through the column in the presence of phenylmethylsulfonyl fluoride 0.001M (PMSF) for about 15 hours at 4° C.

4) The column is then washed three times in order to elute nonspecifically bound plasma proteins from the gel. All washing steps are performed using a Tris-HCl pH 7.5 solution. A 1M NaCl solution is added to the second washing step to elute contaminants.

5) Elution of fibronectin is carried out by using 0.1M Na acetate+1M KBr solution.

6) Two dialysis steps are then performed to eliminate contaminants (Triton X-100, Kbr, Na acetate). Dialysis versus PBS and sterile water are respectively done.

7) Solution is concentrated by ultrafiltration under nitrogen pressure.

8) Terminal sterilizing filtration using a 0.22 μm filter is done to warrant sterility.

9) Fractions are aliquoted and frozen at −20° C. until their incorporation into the topical dosage form.

EXAMPLE 2

Polyacrylic acid gels

Polyacrylic acid (carbomer) gels (Carbopol® carbomer, BF Goodrich) were prepared. Carbomer (carbopol) is a polymer derived from acrylic acid. It is a high molecular weight polymer (740.00 to 5.000.000) that gelifies when neutralized by strong alkalis (NaOH) or amines (triethanolamine). It forms gels at relatively low concentrations, that is as low as 0.25%, and its viscosity is strongly reduced by the addition of electrolytes.

Preferred grade of polyacrylic acid is Carbopol® 934-P carbomer at concentrations ranging from 0.35 to 0.75% (w/w). Lower concentrations are insufficient to promote adherence to the wound and higher concentrations reduce the release of fibronectin from the gel. Viscosity of polyacrylic acid gels is stable between pH 6 to 8 with a preferred pH range between 6.5 to 7.5. Viscosity is reduced in the presence of strong electrolytes.

A polyacrylic acid gel containing (w/w) fibronectin 0.2%, Carbopol® 934-P carbomer 0.375%, and chlorocresol 0.1% was prepared as follows: chlorocresol (1.0 g) was dissolved in warm (65° C.) deionized water (95 ml) under slow agitation. When the chlorocresol is completely dissolved, the solution is cooled at room temperature while maintaining agitation. Carbopol® 934-P carbomer (3.75 g) was then added, dispersing it slowly on the surface of the solution, and mixed with a paddle type stirrer for about 3 hours. This dispersion was then autoclaved to provide a sterile concentrated gel base (3.75% w/w). A stock solution of fibronectin 2.2 mg/ml (90 ml) was filtered through a 0.22 μm acetate filter. A polymerization promoter, sodium hydroxide, was added to the fibronectin solution in an amount that will neutralize a 10 g portion of the Carbopol® carbomer 3.75% dispersion, that is 1250 μl of NaOH 3M. The stock solution of fibronectin and Carbopol® carbomer dispersion were mixed into syringes taking care to avoid introducing air bubbles and contamination in an aseptic environment, such as under a laminar flow hood. Generally, two syringes are used, and multiple exchanges under pressure are applied. An adaptor device, such as a female luer connection can be used to connect the syringes or other exchange apparatus. Vigorous agitation is minimized in order to avoid fibronectin precipitation. This preparation provides a clear, preserved gel (100 g) of fibronectin free from microorganisms with viscosity of about 350.000 cps.

This gel formulation was applied twice a day on leg ulcers in a pilot study in humans and showed an enhanced rate of wound healing without any adverse effect.

In a preferred embodiment, concentrated solutions of fibronectin, up to at least 10 mg/ml, can be prepared. These solutions can then be used to prepare a concentrated fibronectin gel. In order to prepare the fibronectin gel, the following ingredients must be added in sequence, and are required for the preparation of 10 grams of gel, varying in fibronectin concentration from 0.5 to 1.0%. First, the pH of 9.8 grams of demineralized water is adjusted to 11 with 0.094 grams of NaOH 3M. When a carbomer gel is to be prepared, a pH of about 9.0 for the demineralized water is preferred. Lyophilized fibronectin is next dissolved in demineralized water, about pH 9.0 to about pH 11.0 in quantities varying from 0.5 to 1.0 grams. In a final step of the procedure, 0.028 grams of carbomer is added to the mixture.

EXAMPLE 3

Polyoxyethylene-polyoxypropylene block copolymer gels

Polyoxyethylene-polyoxypropylene block copolymer (poloxamer) gels (Pluronic® poloxamer, BASF Wyandotte) were prepared. Preferred grade of poloxamer is Pluronic® F-127 poloxamer at concentrations ranging from 18 to 25% (w/w). Pluronic® F-127 poloxamer is a low molecular weight polymer (2,000 to 13.000) which exhibits thermal gelation characteristics. Gelation occurs when the concentration reaches 18% poloxamer. The viscosity of poloxamer is proportional to the concentration of the polymer, type of polymer used (molecular weight) and temperature. Fluid at 4° C., the polymer gelifies with increasing temperatures, providing high viscosity values at room temperature. In contrast to Carbopol® carbomer, the addition of ions enhances the viscosity of the preparation.

Concentrated aqueous solution (20 to 30%) have been reported to show a dramatic increase in viscosity when heated from 4° C. to body temperature. Furthermore, if the ionic strength of the solution is increased, the viscosity is increased more rapidly with rising temperature. Several grades are available but the F-127 grade is the least toxic and gelation can occur at lower concentrations. Gels of poloxamer prepared in this invention are low viscosity solutions at 4° C. and gelify rapidly when they are warmed to body temperature.

A poloxamer gel containing (w/w) fibronectin 0.2% and Plutonic F-127 poloxamer 20% was prepared as follows: a stock solution of fibronectin 2.2 mg/ml (80 ml) was filtered through a 0.22 μm acetate filter. Pluronic® F-127 poloxamer (20 g) was added to 80 ml of the fibronectin solution and allowed to dissolve without agitation at 4° C. for about 3 days. The resulting solution (100 g) is very liquid-like. Gelation occurs instantly when the solution comes into contact with the wound. A sterilizing filtration process performed at 4° C. could also be applied to the final solution if sterile poloxamer powder cannot be obtained. Viscosity varies from not detectable values at 4° C. to 450.000 cps at room temperature.

EXAMPLE 4

Cellulose derivative gels

Hydroxypropylcellulose (HPC) gels were prepared. In order to illustrate this type of formulations, the preparation

of a HPC 3% gel is described as below. Preferred grade is Klucel-HF at concentrations ranging from 2 to 4% (w/w).

A gel formulation containing (w/w) fibronectin 0.1%, HPC 3% and parabens was prepared as follows: methylparaben (0.05 g) and propylparaben (0.02 g) were dissolved in warm deionized water (94 ml). HPC powder was sterilized by using a dry heat sterilization process. HPC (6 g) was then dispersed in this solution and allowed to be mixed with a paddle type stirrer for about 3 hours. This provides a sterile concentrated gel base (6% w/w). A stock solution of fibronectin 2 mg/ml (50 ml) was filtered through a 0.22 μm acetate filter. Fibronectin solution (50 ml) was then slowly added to a portion (50 g) of this concentrated base using the low-speed shaft of the stirrer. This provides a preserved gel (100 g) with viscosity of about 150.000 cps.

EXAMPLE 5

Cream formulation

A cream formulation containing (w/w) fibronectin 0.1%, sterile cream base (Schering® base, Schering) and chlorocresol 0.1% was prepared as follows: a stock solution of fibronectin 2 mg/ml (50 ml) was filtered through a 0.22 μm acetate filter. Fibronectin solution (50 ml) was then added slowly to a portion (50 g) of the cream base using the low-speed shaft of a stirrer. This provides a preserved cream (100 g) with viscosity of about 70.000 cps.

EXAMPLE 6

Kinetics of release from different topical dosage forms

The effectiveness of each topical formulation to release fibronectin was evaluated using an in vitro diffusion cell system. Permeation studies were all performed on human breast and abdominal deepithelialized skin samples obtained from breast reduction and abdominal lipectomy surgeries. A 8 μm section was removed from the epidermal surface of the skin using a dermatome (1/10,000 scissor scale) and the dermal side was carefully cleaned of any adhering subcutaneous tissues and/or blood vessels. Deepithelialized human skin was used in order to reproduce the pathological condition met in chronic venous ulcers where the epidermis layer is absent.

The diffusion cell system selected consisted of a rigid receptor containing the skin sample, the deepithelialized side facing upwards into the donor compartment and the dermal side facing downwards into the receptor compartment. The receptor compartment was connected to a circulating buffer circuit. The buffer temperature was maintained at 37° C. while the skin surface was at about 32° C. Each analysis was performed on a 0.64 cm^2 skin sample using a 100 μl aliquot of ^{125}I-fibronectin topical formulation specimen. After the experiment, the skin was removed from the diffusion cell, washed 10 times with a 8 ml water volume by wash, and analyzed for its content of radioactivity in a gamma radioactivity counter. The total amount absorbed (dermis+receptor compartment) divided by the dose applied gave the percent absorption.

All dosage forms were made in salt-free solution since viscosity values could have been influenced by the presence of electrolytes. For instance, viscosity values of carbomer gels are reduced in the presence of strong electrolytes in contrast with poloxamer gels which are more viscous when electrolytes are added to the preparation.

The design for the rigid receptor, receptor compartment, and circulating buffer circuit used in the diffusion cell system of the invention can be selected from among the various alternative known to those skilled the art. Sundry examples of diffusion cell systems are described in the percutaneous absorption studies described below. Many diffusion cell systems which can reliably hold the dermal side of the skin sample in contact with the buffer solution and the deepithelialized side in contact with the ambient or heated air may be suitable for use together with the deepithelialized skin sample of the invention. A preferred diffusion cell system is the TDC-01 Teflon® Flow-Thru Diffusion Cell made by Crown Glass Company, Inc. (Somerville, N.J.).

Several authors have compared percutaneous absorption studies using in vitro and in vivo techniques to establish the reliability of results using these methods[13,14,15]. These comparisons have clearly shown that in vitro studies can accurately reflect the living state. Statistic analysis applied to our experiments has demonstrated a good correlation value between studies performed on skin obtained from different sources. These data have shown that the origin of the skin did not have any effect on results.

Percutaneous absorption studies are usually performed on intact skin and are designed to evaluate the release of a substance from a topical vehicle and its absorption through the major cutaneous barrier, that is the stratum corneum. In cutaneous ulcers, the barrier effect of the stratum corneum is absent. With this pathological condition, only the diffusion from the dermatological vehicle will be a major determinant for the ulterior penetration of the drug into the dermis. The diffusion cell system described above is a suitable in vitro model for cutaneous ulcers.

Kinetic data of the release of fibronectin from various dosage forms were obtained at 4, 12, and 24 hours. Table 1 summarizes these data for t=12 hours. The control consisted of ^{125}I-fibronectin in phosphate buffered saline solution, pH 7.4. Liposomes used in the Carbopol® 934 P carbomer (1%)+liposomes (15%) formulation (Lipogel) were made from Proliposomes (Pro-lipo 3090 SH™, Lucas Mayer, France). Cellulose derivatives are identified as CMC for sodium carboxymethylcellulose and HPC for hydroxypropylcellulose. Dermabase® base (Borden, Ltee., Don Mills, Ontario, Canada) and Schering® base are cream bases available on the market and were diluted 1:1 for these experiments. The symbol [ ] refers to components concentration and "Abs value" to the percentage of radiolabelled fibronectin found in the dermis after an exposition time of 12 hours.

TABLE 1

Formulation	[ ]	Abs value
Control		24.75%
Lipogel		3.70%
Dermabase ® base	(1:1)	5.80%
CMC	3%	6.70%
Carbopol ® 934 P carbomer + glycerol (Carbogly)	0.375%/10%	7.80%
Schering ® base	(1:1)	9.90%
Pluronic ® F-127 poloxamer	20%	12.80%
Carbopol 934 P carbomer	0.375%	13.40%
HPC	3%	15.20%

FIG. 1 plots kinetic data of three gel dosage forms and control solution over time. From this graph it can be seen that the absorption process tend to be more important between time 0 and 12 hours than between time 12 and 24 hours, suggesting that two applications per day could release more fibronectin than an once a day schedule.

FIG. 2 depicts cutaneous absorption of radiolabelled fibronectin from various dosage forms and from control at time=12 hours. The Dunnett statistic test was used to identify statistically significant differences between Carbopol® carbomer gel and other formulations. This test has also

shown significant differences between Lipogel. Carbogly. and Carbopol® carbomer gel. results that can be correlated with those of effectiveness obtained during clinical trials (see example 9). The efficacy of the Carbopol® carbomer gel formulation is particularly surprising since Carbopol® carbomer gel has a higher degree of viscosity than many of the other formulations studied. Also noteworthy are the difference in Abs value between the Carbopol® carbomer gel and CMC formulations since they both share the same degree of viscosity.

FIG. 4 show that a clear relationship between viscosity and absorption does not always exist. when considering the some of the preparations for which viscosity values were determined. For instance. Dermabase® base which has a relatively low viscosity (119.000 cps) when compared to Carbopol® carbomer gel (411.300 cps) presents poor release capabilities (5.80%) when compared to Carbopol® carbomer gel (13.40%).

FIG. 5 demonstrates that higher absorption values than 13.40% can be obtained using 0.28% of carbomer and higher concentrations of fibronectin. FIG. 6 directly compares the absorption values obtained in deepithelialized skin diffusion cell system for 0.28% and 0.375% carbomer gels. both of which have 0.2% fibronectin.

In FIGS. 5 and 6. the amount of fibronectin was measured using ELISA procedures. A polystyrene microtiter plate is incubated with 100 µl of different fibronectin samples in 50 Mm carbonate/bicarbonate buffer. pH 9.6 at 4° C. overnight. A solution of 5% bovine serum albumin (BSA) in PBS Tween. pH 7.5 is used as a blocking buffer for 30 minutes at 37° C. The plate is washed four times with PBS Tween pH 7.5 and 100 µl of rabbit anti-FN. produced by methods well known in the art. diluted 1/100.000 in 0.5% PBS Tween pH 7.4. are added and the plate is incubated at 37° C. at 1 hour. After rinsing four times with PBS buffer. 100 µl of the goat anti-rabbit horseradish peroxidase conjugated (Jackson Immunoresearch Laboratories. Inc.. Pa) diluted 1/100.000 in 0.5% PBS Tween pH 7.4. are added and the plate is incubated at 37° C. for 1 hour. Excess conjugate is then thoroughly removed by washing. and the peroxidase fixed to the wells is detected by addition of ABTS (2.2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) in citrate buffer pH 4.6 containing 0.015% hydrogen peroxide. The reaction is followed by increases in absorbancy at 410 nm as compared with a standard fibronectin peroxidase reaction.

EXAMPLE 7

Immunofluorescence study of fibronectin absorption in deepithelialized skin diffusion cell system

In order to determine the localization of fibronectin in deepthialized skin specimens (3 mm thick), an immunofluorescence procedure was performed. After fibronectin control or experimental sample had been allowed to absorb for a period generally of 12–24 hours in deepithelialized skin sections; the deepithelialized skins sections were then frozen. The frozen deepithelialized skins section were sliced into 2 µm frozen skin sections and deposited on glass cover slips. Skin sections were prepared at different skin depths.

The 2 µm frozen skin sections deposited on cover slips were first incubated with anti-human fibronectin mouse monoclonal antibody (Sigma Chemical Co., St. Louis. Mo.). diluted at 1/200 in phosphate buffered saline (PBS). The incubation was conducted for 30 minutes at room temperature. At the end of the incubation. the skin sections were extensively washed with PBS. The skin sections were then incubated with the second antibody. anti-mouse goat Ig fluorescein isothiocyanate (FITC) conjugated antibody (Sigma Chemical Co., St. Louis, Mo.). diluted at 1/50 in phosphate buffered saline (PBS). The incubation was again conducted for 30 minutes at room temperature. and the skin sections were extensively washed with PBS at the end of the second incubation.

After the second antibody incubation. cover slips were mounted in an aqueous mounting medium containing phosphate buffered glycerol. Skin sections were examined with a Leitz photomicroscope equipped for epifluorescent optics.

FIG. 7 depicts the immunofluorescence of fibronectin which can be detected in 2 µ frozen deepithelialized skin section after an absorption time of 12 hours in a diffusion cell system. FIG. 7A is a negative control of 0.5% fibronectin solution prepared as described in the last paragraph of Example 2. FIG. 7B is 0.5% (w/w) fibronectin in Carbopol® carbomer gel at around 482 µm skin depth. and FIG. 7C is 0.5% (w/w) fibronectin in Carbopol® carbomer gel at around 2000 µm skin depth.

EXAMPLE 8

Stability of fibronectin in gel

Biological activity and integrity of the macrostructure of fibronectin in gel formulations were evaluated (FIG. 3). Assays were performed on a specimen of gel containing (w/w) fibronectin 0.2%. Carbopol® P-934 0.375% carbomer. and chlorocresol 0.1%. The specimen had been kept at 4° C. for 32 weeks.

Electrophoresis techniques were used in order to determine the integrity of macrostructure of fibronectin in gel. After the specimen of gel was dissolved in 1M NaCl+Tris-HCl pH 6.8–7.4 solution. it was allowed to migrate on an 7.5% acrylamide gel according to the method of Laemmli ("Denaturing (SDS) discontinuous gel electrophoresis: Laemmli gel method," pages: 10.2.4–10.2.9, *Current Protocols in Molecular Biology* 1994). Compared with a fresh standard solution (column 0), results showed that close to 100% of the fibronectin can be identified around the 220.000 (column B) band indicating that very little. if any. degradation occurs.

Biological activity was evaluated using an affinity chromatography test. Gelatin-binding is one of these biological activities that can be assessed with relative ease. After a specimen of gel was dissolved in a 1M NaCl solution. a known amount of this viscous solution was placed in an Eppendorf tube in the presence of gelatin-Sepharose 4B and then vortexed. The content was further rinsed with a fresh 1M NaCl solution. centrifuged and the supernatant discarded in order to eliminate contaminants such as Carbopol® carbomer and chlorocresol that came from the dissolution of the gel. Fibronectin was eluted from the gelatin-sepharose 4B using a 1M KBr solution. The fraction collected was allowed to migrate on an 7.5% acrylamide gel according to the method of Laemmli. The band was then evaluated with respect to its content of fibronectin using a densitometric scanning assay. The specimen collected could also be evaluated spectrophotometrically using optical density at wave length λ=280 nm.

Compared with a freshly prepared gel of fibronectin (column 0), it can be seen that a large amount (80%) of fibronectin was recovered from the specimen of gel formulation (column 8 months) indicating that gelatin-binding activity of the glycoprotein can be preserved for a long period of time in this dosage form.

EXAMPLE 9

Clinical trials: treatment of chronic leg ulcers

We have conducted four clinical trials (pilot studies) to investigate the usefulness of different dosage forms containing exogenous human plasma fibronectin in the treatment of

chronic venous ulcers of lower limbs. In these trials autologous plasma fibronectin was used and patients with ulcers that were resistant to the conventional therapy for at least three months were selected.

The specific objective of the first experiment was to determine the effectiveness of topically applied fibronectin as a wound healing promoter. Seven patients were included in this study and were instructed to "flood" the wound area with a solution of fibronectin 1 mg/ml (0.1%) in PBS (phosphate buffered saline) twice a day. After two months of regular application of that solution, five of these patients presented with a dramatic decrease in their wound size, specifically at least 75% reduction of the integrated surface area.

A second experiment was designed to evaluate the effectiveness of a semi-solid dosage form which contained by weight fibronectin 0.1%, encapsulated in liposomes 15%, which in turn were incorporated in Carbopol® carbomer (1%) formulation known as Lipogel. Hypothesis was that if the contact time of the glycoprotein with the wound could be enhanced, a more rapid decrease in the healing time could theoretically be observed. Six patients were included in this study and they had to apply the formulation to their wound twice a day. None presented a substantial decrease of their wound size during the following three months of regular treatment.

In an attempt to improve the dosage form, an experiment was undertaken to evaluate the therapeutic potential of a topical gel formulation containing (w/w) fibronectin 0.2% incorporated in Carbopol® carbomer 0.375% and glycerol 10% (Carbogly). Glycerol had been added to the formulation in order to take advantage of its humectant effect which could be beneficial to the wound. Eleven patients were recruited for this study and they also had to apply the gel twice a day. Among these patients, 27% had a regression of more than 50% of their wound size after three months of treatment.

Results from the permeation studies may explain, at least in part, what could have occurred in previous experiments. FIG. 2 shows that preparations such as Lipogel and Carbopol® carbomer+glycerol do not lead to high absorption values. In contrast, Carbopol® carbomer 0.375% without glycerol provides significantly higher absorption values ($p<0.001$). The solution used in the first experiment is identified as the control in this graph. This last preparation provides the highest release capabilities, but it does not represent a formulation that could be useful to patients owing to its fluid consistency.

Considering these results, a formulation containing fibronectin 0.2% (w/w) in Carbopol® carbomer 0.375%, without glycerol was investigated in eight patients. According to clinical and permeation studies, this formulation is the preferred carrier using Carbopol® carbomer that is available for the use of fibronectin in topical wound healing. Preliminary data showed that 50% of patients studied presented a regression of more than 50% of their wound size within three months of treatment, including two complete responses (100% healing) that occurred within the first eight weeks of treatment. The present invention also provides other useful wound healing formulations that can be selected using the permeation studies described in Example 6.

EXAMPLE 10

Case reports

To illustrate the efficacy of the formulation containing fibronectin and Carbopol® 934-P carbomer-0.375% (w/w), we present two specific cases of chronic venous leg ulcer. These cases are of interest in that the first case was highly resistant to conventional therapy and the second case was a large ulcer. Factors such as duration and surface area have been identified by several authors as playing a major role in the prognosis of the venous ulcer.

Case 1

A 37-year-old woman presented with a ten-year history of chronic venous ulcer of the right lower limb. Her medical history was not significant except for four episodes of phlebitis. The last episode occurred during pregnancy and ultimately resulted in an ulcer. Review of medical treatments that were tried revealed the use of topical antiseptics, elastic stockings, and skin grafting without any positive result.

The patient presented to our clinic with a 1.60 cm^2 aching wound. Despite the fact that her ulcer was relatively small, it appeared highly resistant to therapy. Six weeks after starting the application of the gel of fibronectin, a 92% reduction of her wound size could be observed. Complete reepithelialization was noted after a ten-week course of treatment. A follow-up visit scheduled one month later revealed no deterioration in her wound condition.

Case 2

A 39-year-old man presented with a seven-month history of chronic venous ulcer of the left leg. His medical history was not significant except for a saphenectomy of the left lower limb twelve years before. Topical antibiotics were prescribed to the patient without any effect on his wound size.

He presented to our clinic with a 10.5 cm^2 ulcer resulting from a local trauma. Lymphedema of the left lower limb was important and a large crusty necrotic layer bordered the wound. The patient's occupation constrained him to remain standing for long periods of time. Although this situation probably worsened his wound condition, it could not be eliminated.

After four weeks of regular application of a placebo gel and normal saline, the wound size increased to 21.5 cm^2 as a consequence of local debridement. The placebo gel comprised 0.375% Carbopol® 934-P carbomer, 0.1% chlorocresol, purified water and NaOH to adjust the pH. Active treatment with the fibronectin-containing Carbopol® carbomer gel formulation was begun at this time. Maximum wound size was noted six weeks later (37.5 cm^2), revealing a larger ulcer than initially assumed. The wound healing process took place between six to eight weeks and was completed after 31 weeks of active treatment. A follow-up visit scheduled one month later revealed no deterioration in his wound condition.

Although the present invention has been described in relation to particular embodiments thereof, many other variations and modifications and other uses will become apparent to those skilled in the art. It is therefor understood that numerous variations of the invention can be made which are well within the scope and spirit of this invention as described in the appended claims.

REFERENCES

1—Hynes, R. O., Methods for identification of fibronectin (chap. 2, page 12), IN: *Fibronectins* New-York: Springer-Verlag, 1990.

2—Hynes, R. O., Methods for identification of fibronectin (chap. 2, pages 7–23) and Wound healing, inflammation, and fibrosis (chap. 14, pages 349–64), IN: *Fibronectins* New-York: Springer-Verlag, 1990.

3—Brotchie, H., Wakefield, D. Fibronectin: Structure, function and significance in wound healing. Australas J Dermatol 1990; 31:47–56.

4—Nishida, T., Nakagawa, S., Awata, T. et al., Rapid preparation of purified autologous fibronectin eyedrops

from patient's plasma. Jpn J Ophthalmol 1982;26:416–24.

5—Phan, T. M., Foster, C. S., Boruchoff, S. A. et al., Topical fibronectin in the treatment of persistent corneal epithelial defects and trophic ulcers. Am J Ophthalmol 1987;104: 494–501.

6—Wysocki, A., Baxter, C. R., Bergstresser, P. R. et al., Topical fibronectin therapy for treatment of a patient with chronic stasis ulcers. Arch Dermatol 1988; 124:175–77.

7—Edwards, C. A., Piet, M. P. J., Chin, S. et al., Tri (n-butyl) phosphate/detergent treatment of licensed therapeutic and experimental blood derivatives. Vox Sang 1987; 52:53–9.

8—Horowitz, B., Bonomo, R., Prince, A. M. et al. Solvent/detergent-treated plasma: a virus-inactivated substitute for fresh frozen plasma. Blood 1992; 79: 826–31.

9—Grinnell, F., Ho, C. H., Wysocki, A., Degradation of fibronectin and vitronectin in chronic wound fluid: Analysis by cell blotting, immunoblotting, and cell adhesion assays. J Invest Dermatol 1992; 98: 410–6.

10—Chen, W. Y. J., Rogers, A. A., Lydon, M. J., Characterization of biologic properties of wound fluid collected during early stages of wound healing. J Invest Dermatol 1992; 99: 559–64.

11—Berman, M., Manseau, E., Law, M. et al., Ulceration is correlated with degradation of fibrin and fibronectin at the corneal surface. Invest Ophthalmol Vis Sci 1983; 24: 1358–66.

12—Horowitz, B., Chang, M. D. Y., Preparation of fibronectin for therapeutic administration. IN: D. F. Mosher (ed.), *Fibronectin*, pages 441–55, San Diego, Academic Press 1989.

13—Franz, T. J., Percutaneous absorption. On the relevance of in vitro data. J Invest Dermatol 1975: 64:190–95.

14—Bronaugh, R. L., Stewart, R. F., Congdon, E. R. et al. Methods for in vitro percutaneous absorption studies. I: Comparison with in vivo results. Toxicol Appl Pharmacol 1982;62:474–80.

15—Bronaugh, R. L., Stewart, R. F., Methods for in vitro percutaneous absorption studies IV: The flow-through diffusion cell. J Pharm Sci 1985: 74:64–67.

I claim:

1. A method for selecting formulations containing an active agent, which formulations impede the absorption of said agent by broken skin, comprising measuring the absorption by deepithelialized skin of a premeasured amount of an active ingredient contained in a topical formulation by:

a) applying the topical formulation containing a premeasured amount of an active ingredient to the deepithelialized side of a deepithelialized skin sample, said skin sample being contained in a deepithelialized skin diffusion cell system comprising a donor compartment and a receptor compartment, said skin sample being placed so that the deepithelialized side faces into the donor compartment and the dermal side into the receptor compartment;

b) measuring the amount of active ingredient in the deepithelialized skin sample and in the receptor compartment after a period of time: and

c) determining the Abs value of the topical formulation by dividing the amount of active ingredient measured in step b) by the amount applied to the deepithelialized skin sample in step a).

2. The method according to claim 1, wherein the deepithelialized skin sample is a human skin sample from which a $^{8}\mu$m section of the epidermal surface has been removed.

3. The method according to claim 1, wherein the receptor compartment is connected to a circulating buffer circuit containing a buffer solution.

4. The method according to claim 1, wherein the buffer solution is maintained at 37° C. and the deepithelialized skin side is maintained at 32° C.

5. The method according to claim 1, wherein the deepithelialized skin sample is obtained from a species which serves as an animal model for human skin.

6. The method according to claim 5, wherein the species is selected from the group consisting of human being, domestic pig, laboratory rabbit, laboratory guinea pig, and laboratory rodents.

7. The method according to claim 1, wherein the deepithelialized skin sample is obtained from a domestic, laboratory or companion animal.

8. The method of claim 1, wherein the active agent is prepared in a salt-free solution.

9. The method according to claim 1, wherein the period of time in step b) is about 12 hours and the active ingredient is fibronectin.

* * * * *

History and clinical use of alginate

The application of algae in the treatment of wounds was reported as early as in the 18th century as a remedy for healing the skin after blisters. An early assessment of the use of alginates as hemostats and wound dressings was made by Blaine (1), who also stated their lack of toxicity.

In 1948, other more general areas of clinical usage were described, such as the results from a three-months trial on the use of alginate in the casualty department of Croydon General Hospital. The study was reported by Bray et al. (2) where it was stated that alginates in the form of films, wool, gauzes and clots were applied to a wide range of wounds, including burns, lacerations, trophic ulcers and amputations and, in all cases, healing was found to be rapid and uneventful.

Alginate fiber is used in the treatment of burns and donor sites, where the hemostatic and absorbent properties of the material should be at their most useful. Groves and Lawrence (3) have described a study in which they compared an alginate dressing with a standard gauze pad. They found that in a simple laboratory test, the alginate absorbed nearly three times as much citrated blood as the gauze.

Attwood made an investigation of the effect of longer term application of alginates to donor sites (4). He found that sites treated with an alginate dressing healed more rapidly than those treated with paraffin tulle. The quality of healing, as well as patient compliance, was also reported to be significantly better with the alginate dressing.

Properties of alginates in wound healing

Once the dressing, film or powder is in place, exudate is absorbed to form a hydrophilic gel over the surface of the wound, providing a moist environment for wound healing. The properties of the gel and the rate at which it is formed differ from product to product, being a function of the source of the alginate raw material, the method of production and the ratio of Na/Ca in the product.

If the wound healing material is applied to a dry, non-exuding wound, there is little advantage to be gained. This is based upon the fact that alginates depend for their activity upon the formation of a gel by the absorption of wound exudate. As long as the alginate is applied to exuding wounds, the material shows no adherence. If however the material is applied to a dry wound, part of the alginate material may be incorporated into the granulating tissue and removal

The application of algae in the treatment of wounds was reported as early as in the 18th century as a remedy for healing the skin after blisters.

Studies show that sites treated with an alginate dressing healed more rapidly than those treated with paraffin tulle.

PRONOVA BIOPOLYMER

The alginate gel can serve as vehicle or carrier for the application of drugs to wounds as well as having a protective function.

may be cumbersome. When the latter is the case, the alginate should be removed by the use of saline which facilitates an exchange from the insoluble calcium gel to the soluble sodium gel.

The biodegradability of alginate fiber residues has been the subject of recent debate (5), and, although it is generally assumed that the polysaccharide molecule is broken down to its monomers by enzymatic activity within the body, this has not been confirmed experimentally.

When applied as a calcium gel onto the wound (6), the gel dries to an adherent, non-toxic pliable protective film. The gel is compatible with other medicaments, hence it can serve as a vehicle or carrier for the application of drugs to wounds as well as having a protective function (7).

Another important feature of calcium alginate as a wound healing material is its ability to arrest hemorrhage (2,7). This property is the main reason for the widespread application of alginate fiber dressings in the dental and medical surgery. The exact mechanism for the hemostatic effect is not fully characterized, but the calcium released from the alginate will activate the blood coagulation cascade and stimulate platelet aggregation.

Another theory explaining the hemostasis is based on the mechanical effect that occurs during gelling, which provides a matrix for blood coagulation.

The production of calcium alginate fiber

By extruding a 5 - 10 % solution of sodium alginate into a spinning calcium chloride bath, instantaneous gelling will take place. Depending upon the pressure applied during the extrusion process and the size of the many orifices of the extruder, a vast range of filaments as far as thickness and strength may be produced.

The filaments may then be hardened through additional contact with calcium chloride and they are eventually run over rollers and exposed to heat in order to remove excess moisture.

Another important feature of calcium alginate as a wound healing material is its ability to arrest hemorrhage.

The technique described above is today commercially applicable, and has been patented by different manufacturers (8,9). The calcium alginate fibers are subsequently applied in the manufacture of alginate fiber dressings; comprising woven, non-woven and gauze types.

Recommended alginates for dermatology and wound healing

The alginates shown in the table II-1 are tailor-made for dermatology and wound healing.

Alginates high in guluronic acid result in strong and brittle fibers which form highly absorbent gels, whereas alginates high in mannuronic acid result in softer fibers which form into less absorbent gels. Both alginates rich in high guluronic acid and alginates rich in high mannuronic acid are applied in commercially available products today.

Table III-1 . *Measured on Brookfield Viscometer, 1 % solution, 20°C . **British Standard.

TRADE NAME	SALT FORM	VISCOSITY* mPas	GULURONIC ACID, %	MANNUR. ACID, %	MESH SIZE**
PROTANAL LF 10/60	Na	10 - 70	65 - 75	25 - 35	60
PROTANAL LF 10/40	Na	10 - 40	65 - 75	25 - 35	40
PROTANAL LF 120M	Na	70 - 150	35 - 40	55 - 60	120
PROTANAL TFX 200	Ca	100 - 400	65 - 75	25 - 35	200

References

1. Blaine, G. Experimental observations on absorbable alginate products in surgery, *Ann.Surg.*, 194, 125, 102-114.
2. Bray, C. et al. New treatment for burns, wounds and haemorrhage, *Nurs. Mirror*, 1948, 86, 86, 239-242.
3. Groves, A.R. and Lawrence, J.C. Alginate dressing as a donor site haemostat, *Ann.R., Coll. Surg.*, 1986, 68, 27-28.
4. Attwood, A.I. Calcium alginate dressing accelerates graft donor site healing. Paper presented to the Winter Meeting of the British Association of Plastic Surgeons, England, Dec. 1986.
5. Schmidt, R.J. and Turner, T.D. Calcium alginate dressings, (letter), *Pharm.J.*, 1986, 236, 578.
6. Protective gel composition for wounds. United States Patent 4.395.045, Mason, Jr. et al. Jul. 5, 1985.
7. Blaine, G. A comparative evaluation of absorbable haemostatics. *Post-Graduate Medical Journal*, Vol. XXVIII, p. 613.
8. Procède de la fabrication de fibre d'alginate de calcium. Demande de brevet dinvention No 78, 00142, Institute Nationale de la Propriete Industrielle, 03.03, 1978.
9. Process for treating alginic materials. International Application published under the Patent Cooperation Treaty (PCT), 27.09, 1984.

PRONOVA

PRODUCT SPECIFICATION

PRONOVA BIOPOLYMER

Name: PROTANAL LF 10/60 14328230/0196

Product: SODIUM ALGINATE

Product Grade: PHARMACEUTICAL

TYPE OF ALGINATE:	Highly refined sodium alginate from brown seaweed.
REGULATORY COMPLIANCE:	European Pharmacopoeia

CHEMICAL AND PHYSICAL CHARACTERISTICS

STRUCTURE:	Guluronic acid: 65 - 75% Mannuronic acid: 25 - 35%
APPEARANCE:	White to yellowish powder, almost tasteless odorless
VISCOSITY (in 1% solution):	20 - 70 mPa.s
pH (in 1% solution):	6.0 - 8.0
LOSS ON DRYING:	Max. 15.0%
PARTICLE SIZE:	Min. 99% thr. 60 mesh BS (0.250 mm)
SULPHATED ASH (600°C):	30 - 36%
INSOLUBLES:	Max. 0.2%
CALCIUM:	Max. 0.5%
CHLORIDES:	Max. 1.0%
TOTAL HEAVY METALS:	Max. 20 ppm
ARSENIC (As);	Max. 3 ppm
LEAD (Pb):	Max. 10 ppm
IRON (Fe):	Max. 300 ppm

MICROBIOLOGY:	Total viable count: max. 5.000/gram E. coli: negative Salmonella: negative Mould and yeast: Total viable count less 200 cfu/g

PACKING:	25 kg plastic reinforced paper bag, PE-lined
STORAGE.	Should be stored in a dry and cool place

METHODS OF ANALYSIS ARE SUPPLIED UPON REQUEST

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Promise and Problems Loom for Stem Cell Gene Therapy

By Paul Smaglik

Stem cell transplantation and stem cell gene therapy share the same promise—and many of the same problems.

The promise looms so large that gene therapy researchers and transplant scientists alike often refer to stem cells as the "ultimate target."[1] In the transplantation field, such progenitor cells purified from either a donor or the patient could theoretically improve the efficacy of the procedure and reduce the risk of graft versus host disease (GVHD).[2] And in gene therapy, transducing a progenitor cell should give rise to an unlimited number of similarly "fixed" cells, potentially curing single-gene disorders.

The problems, though, remain formidable, notes James Wilson, director of the Institute for Human Gene Therapy at the University of Pennsylvania, and the president of the American Society for Gene Therapy (ASGT). Wilson has been warily watching the progress of stem cell research in both fields, but he has yet to take the plunge in his own lab. "One of the problems with using cell transplantation to repopulate an organ is the cells that exist there have to get out of the way," he notes. That means stem cell treatments would theoretically involve eliminating muscle to cure Duchene's muscular dystrophy or creating massive liver damage to heal hemophilia. Bone marrow transplants, which require such cytoablation, have a 10 percent mortality rate, he notes. And even if the high doses of chemotherapy and radiation necessary to kill the existing bone marrow don't also kill the patient, they greatly weaken him or her, limiting the utility of such transplant procedures.

Wilson notes that cytoablation causes "the same problems" in stem cell gene therapy. Repopulation is "going to be a major issue," he predicts. Curiously, Wilson sounds confident about overcoming one obstacle in stem cell gene therapy, getting a retroviral vector to enter cells that seldom divide. "We could get the genes into those cells. There's no doubt in my mind. But I think it's premature to get too excited about genetically modifying a human stem cell until we can figure out how to control its [illegible] and differentiation."

Fortunately for both fields, problems facing both may also [illegible] the same solutions—solutions that are growing tantalizingly closer to resolution, according to Curt I. Civin, Johns Hopkins University oncologist, and Harry L. Malech, deputy chief of the Laboratory of Host Defenses at the National Institute of Allergy and Infectious Diseases. Civin is primarily interested in stem cell–based transplants to treat cancer, and Malech is in the midst of a stem cell gene therapy trial to treat a single-gene disorder.[3,4] "They each inform the other," Malech notes of the two fields. Indeed, on June 30 Civin received an "Inventor of the Year" award from the Intellectual Property Owners Association, for developments that help both. In 1984 Civin developed the CD34 monoclonal antibody that binds to antigens only present in hematopoietic stem cells. Those cells give rise to blood, marrow, and immune cells. More recently, he developed a process to purify hematopoietic stem cells on a larger scale. Those two discoveries have helped solve a problem key to both fields—isolating the target cells, which make up about 1 percent of bone marrow cells. That step represents the foundation on which both stem cell gene therapy and transplantation can be built.

A network of gas-permeable plastic bags connected by tubing helps make ex vivo stem cell gene therapy more feasible. The insides of the bags are coated with recombinant human fibronectin fragments. That protein helps more retroviruses carrying therapeutic genes to enter the targeted stem cells, which are also grown in bags. The tubing allows growth factors to be pumped easily into and out to the network. The bags also maximize the interaction between stem cell, virus, and fibronectin.

The next step, making stem cells differentiate into specific cell types, is close to being solved, Civin claims. "If you gave me a pot of CD34 positive cells, I could make it so that, by giving them the right growth factors and conditions, most of the cells become either white cells, or red cells, or platelets." However, for stem cells to be more useful in either procedure, researchers must find a way to make stem cells beget stem cells. "We don't quite yet have the self-renewal problem licked," Civin explains. "We're getting there." The success of stem cell gene therapy depends on that problem's resolution for two reasons. First, even the best viral vector will likely only enter a small fraction of stem cells, so growing more would result in a bigger pool. And second, because viral vectors can only enter cells while they are dividing, making them grow would unlock the door to transduction.

Still, Civin suspects that problem, too, is on the threshold of resolution. Researchers, including Malech, are getting some of the cells to divide at least once, allowing retroviral vector entry. "The trick is to use optimal combinations of cloned recombinant human growth factors and stem cells in special media." Using that approach—and a painstakingly complex protocol (see related story, below), Malech achieved subtherapeutic levels of transduction in a single-gene disorder. He transduced about 0.5 percent of targeted cells in two of four patients with the rare immune deficiency chronic granulomatous disease (CGD), Malech reported at last month's ASGT meeting in Washington, D.C. "We're at the borderline of most peoples' definition of a permanently engrafting stem cell." He estimates that they need a minimum long-term level of 5 percent correction to lessen the disease's impact. "We have to boost it a lot more," Malech says of the existing transduction level. Many animal models have already yielded higher levels of expression, but researchers destroyed existing bone marrow to achieve those results.[3,4]

The transplantation field again points to a gentler alternative. Malech discussed an experiment applying that approach to people during a recent Lake Tahoe, Nev., stem cell conference. Physicians isolated stem cells from CGD patients' siblings with matching tissue type, then purified them. Meanwhile, they gave the patient mild marrow suppression and immune suppression, rather than complete bone marrow ablatement. The patients then

For Stem Cell Transduction, The Solution Is in the Bag

Building a platform for stem cell gene therapy required timing, technology—and a bit of luck, notes Harry L. Malech, deputy chief, Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases.

The treatment begins by mobilizing patients' hematopoietic stem cells from their bone marrow into their blood. In his trial for chronic granulomatous disease (CGD), Malech used granulocyte macrophage-colony stimulating factor (GM-CSF) and Flt-3 ligand, a novel experimental bone marrow-stimulating growth factor, both provided by Immunex of Seattle. "It greatly raises the number of stem cells in the blood," Malech [illegible] of the experimental combination.

After seven or eight days of treatment with the cocktail, the patient's level of stem cells rises to a harvestable amount. Then a machine plucks the mononuclear cells from the patient but returns the plasma, platelets, and red blood cells. After this apheresis, the blood monocytes, now in plastic bags, are further processed by an Isolex 300i machine provided by Nexell Therapeutics of Irvine, Calif. That machine uses immunomagnetic beads together with antibodies against CD34 and spinning chambers, to separate the stem cells from the rest of the blood product. "After two or three hours, you end up with the waste cells in one bag and purified CD34 cells in another bag," Malech notes.

Those purified CD34 cells are immediately pumped into a new set of special gas-permeable plastic bags, provided by Nexell. Similar bags have been used in earlier gene therapy experiments, but only to grow lymphocytes. The new Nexell bags differ in chemical composition and charge, and therefore are conducive to growing stem cells. Those bags, Malech notes, make the protocol feasible. The bags have insides coated with recombinant human fibronectin fragments (RetroNectin) CH-296 from Takara Shuzo of Otsu, Japan. The product boosts levels of retrovirus-mediated gene transfer.

When Malech heard of the product's transduction-enhancement properties several years ago, he was worried that he would have to abandon his system of bags, which makes handling the cells much easier. "I purchased some of that stuff and it really worked well in my system. I then demonstrated that it worked in the bags the same way it worked in a tissue culture flask. You get a molecular adherent layer that sticks to the inside of the bag." Other growth factors, including the Flt-3 ligand, as well as a purified human serum component, are pumped into the bags. The RetroNectin and the growth factors essentially help force the retroviral vector into the stem cell. "Retroviruses will not go into anything except dividing cells," he notes. "We want to get them [dividing] just long enough to get genes into them, but not hold them in culture too long before they go back into the patient."

After a day of growing, lab workers drain the old media and pump in a new mixture, this time containing the retrovirus, which holds a corrective gene for CGD. The bags again play a key role here, because they allow for a maximum amount of surface area for the vector and the cells to interact—essential for optimal transduction. Researchers then repeat that process for several days, hoping to bump up the level of transduction each time. After four days, transduced cells can be detected on the bag's surface with a microscope. The insides of the bags are vigorously rubbed together, since some of the transduced cells cling tenaciously to their inside surface. Finally, the transduced cells are spun down and washed several times in a salinelike solution fit for infusion. About 30 to 50 milliliters are infused into the patient in 15–20 minutes—one of the quickest parts of the protocol. Without the bags, the protocol would be even more difficult to perform—and likely less dependable. "You can imagine if you're doing this all in flasks that there would be a lot of pipetting in and out, that now is replaced by running fluid through tubing."

He expects that other gene therapy researchers, including Indiana University pediatric hematologist David Williams, who pioneered the use of RetroNectin in Indianapolis, will use similar systems.

—*Paul Smaglik*

For More Information

Takara Shuzo
www.takara.co.jp/english/bio_e/index.htm

Nexell Therapeutics
www.nexellinc.com

Immunex
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INVESTIGATIONAL BROCHURE

Fibronectin semi-solid (hydrogel) and solid (calcium-alginate) wound dressings developed for the treatment of chronic skin wounds

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DERMACOR

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SYNOPSIS

1- This investigational brochure describes the experimental data that form the basis for the final phase of development of novel topical pharmaceutical formulations to be used for wound healing.

2- The topical pharmaceutical formulations embody fibronectin as the active wound healing ingredient. This plasma glycoprotein has been shown to have a number of biological activities that are important for wound healing.

3- The formulations described maximize the contact time of fibronectin to the wound and control the release of this glycoprotein into the wound. Based on extensive laboratory experimentation using a novel approach developed for measuring the release of fibronectin from the topical preparations and the absorption into deepithelialized human skin samples, a semi-solid (hydrogel) and a solid (calcium-alginate) wound dressing were developed.

4- The semi-solid dressing is a carbomer hydrogel containing up to 1% (w/w) of fibronectin, delivering into the wound approximately 13 µg of fibronectin per mm^2 of wound suface area. The solid wound dressing is composed of calcium alginate and contains up to 65% (w/w) of fibronectin, delivering approximately 34 µg of fibronectin per mm^2 of wound surface area.

5- In both the semi-solid and solid wound dressing formulations, fibronectin was found to retain optimal structural and functional stability.

6- Both formulations were shown to stimulate new granulation tissue formation in the rabbit ear acute dermal ulcer model.

7- Purified, solvent/detergent-treated and pasteurized fibronectin can be considered as a safe blood derivative for therapeutic use.

8- A pilot, randomized and placebo-controled clinical trial was conducted in a small group of patients with chronic venous leg ulcers, comparing a carbomer hydrogel containing no fibronectin to a carbomer hydrogel embodying 0.2% fibronectin. This preparation delivered in the wound 0.4 µg of fibronectin per mm^2 of wound surface

area. This concentration in fibronectin was used because at the time of the study, the technology to produce hydrogels with higher concentrations was not available. All patients entered had a venous leg ulcer of at least three months duration and had failed to respond to at least two different treatment modalities.

9- Outcome of the clinical trial: No adverse event was found to be related to the 0.2% fibronectin carbomer hydrogel. Efficacy data analysis, from a statistical standpoint of view, was not reliable given the small sample size. Twenty patients were in the 0.2% fibronectin carbomer hydrogel group and 9 patients formed the carbomer hydrogel alone group. A comparable proportion of patients had at least an 80% decrease in wound size in both treatment groups: 10/20 for the active treatment group and 5/9 in the control group. However, in these responding patients, chronicity of wound at study entry in the fibronectin group was much higher than in the control group. Mean duration of wound prior to randomization was 13.4 ± 10.3 months for the fibronectin group and only 4.6 ± 1.2 for the control group. Since chronicity of wound is likely to be an important factor negatively affecting a treatment response, the positive response of patients with very chronic wounds in the fibronectin group supports the hypothesis that fibronectin can be beneficial for wound healing. It is also possible that wounds of low chronicity, such as that observed in the control group, are more prone to heal spontaneously, making treatment outcome in both groups difficult to compare. Furthermore, by providing a moist and absorbent environment to the wound, hydrogels alone could have some beneficial effect, making the population of patients treated with carbomer hydrogel alone not a true placebo control group. Finally, it must be stressed that the amount of fibronectin delivered in the wound (0.4 µg of fibronectin per mm^2 of wound surface area) could have been suboptimal and that a greater proportion of patients might have responded to a higher dose.

10- Other clinical trials are planned. These trials will be conducted with larger sample sizes, using formulations that deliver up to 34 µg of fibronectin per mm^2 of wound surface area.

INVESTIGATIONAL BROCHURE

INTRODUCTION

Fibronectin is a ubiquitous extracellular glycoprotein containing around 5% carbohydrate. It exists in a soluble form in body fluids and in an insoluble form in the extracellular matrix. Fibronectin plays a major role in many important physiological processes, such as embryogenesis, hemostasis, thrombosis and wound healing (Potts and Campbell, 1994). The characteristic form of plasma fibronectin is a disulfide-bonded dimer of 440,000 daltons, each subunit having a molecular weight of about 220,000 daltons. Plasma fibronectin is also known by various other names, including cold-insoluble globulin, antigelatin factor, cell attachment protein, cell spreading factor, and opsonic a2-surface binding glycoprotein. These names reflect biological activities of fibronectin such as cell recruitment, opsonization of particulate debris, and promotion of wound contraction. Reviews on structure and activities of fibronectin have been published elsewhere (Hynes, 1990).

Wound healing is usually divided into three phases: the inflammatory phase, the proliferative phase, and the remodeling phase. Fibronectin has been reported to be involved in each stage of the wound healing process, particularly by creating a scaffold to which the invading cells can adhere. Initially, there is a release of many mediators to the wound site such as fibronectin and fibrinogen. Fibronectin promotes inflammatory cell migration into the wound and debris phagocytosis by monocytes. Thereafter, angiogenesis and reepithelialization take place. At this stage, fibronectin exerts chemotactic activity on endothelial cells, and promotes epithelial cell and fibroblast migration onto the basal membrane. Fibronectin also appears to be an essential component of the remodeling phase where it plays a major role in the organization of collagen fibrils. The fibrillar collagen ultimately forms fibrous bundles that greatly enhance the tissue tensile strength, leading to wound closure. Normally found in plasma at a concentration of about 300 µg/mL, fibronectin is extracted and purified using a method developed by Horowitz and Chang (1989).

Topically applied plasma fibronectin has been reported as being useful for increasing the rate of wound healing such as in corneal wounds (Nishida et al., 1982; Phan et al., 1987) and leg ulcers (Wysocki et al., 1988). However, there is no suitable topical carrier for use in treating wounds that can ensure delivery of an effective amount of

fibronectin in a pharmaceutically acceptable formulation. A major limiting factor in developing an effective topical dosage form of a drug is not only having an active drug, but also having a formulation that allows the passage of the active drug from the carrier into a site of delivery.

Topical formulations which maximize the contact time of fibronectin to the wound and that control the release of this glycoprotein into the wound have been developed and are described in this investigational brochure.

1. DESCRIPTION OF THE DRUG SUBSTANCES AND THE FORMULATIONS

1.1 Semi-solid fibronectin carbomer hydrogel f rmulation

The first drug substance developed is a hydrogel formulation to be used for the delivery of a potentially effective wound healing amount of fibronectin to a wound site. The hydrogel formulation comprises a water soluble, pharmaceutically acceptable polymer to which can be stably embodied increasing concentrations of fibronectin.

In drug delivery, the term hydrogel is typically reserved for polymeric materials that can absorb a significant amount of water (> 20% of its dry weight) while maintaining a distinct three-dimensional structure (Gehrke and Lee, 1990). The most important characteristic of a hydrogel is its degree of swelling in water. Hydrogels mimic living tissue more closely than any other non-natural material. Their immediate ressemblance to tissue is in their soft, flexible nature and high water content. This helps minimize mechanical irritation and damage to body tissues. Other advantages of hydrogel formulations include: ability to keep the wound moist which results from their high water content, ability to absorb excess water (exudate) in the wound, ease of application to and removal (by washing) from the wound. They also provide a cool feeling when topically applied, a property that can increase patient comfort.

Hydrogels have four major properties: swelling degree, biocompatibility, permeability and swelling kinetics. Example of such compounds include: vinyl polymers (e.g. polyacrylic acid), cellulose and cellulose derivatives. Polyacrylic acid polymer, also referred to as carbomer or Carbopol® (BF Goodrich) was chosen over other polymers (e.g. cellulose and cellulose derivatives), because it was shown to be superior to other pharmaceutically acceptable formulations in the delivery of fibronectin to skin wounds (data presented in section 2).

The drug substance is therefore a hydrogel formulation containing a water soluble, pharmaceutically acceptable polyacrylic acid polymer which is prepared with varying amounts of fibronectin.

1.1.2 Preparation of carbomer hydrogel containing fibronectin

Based on extensive experimentation (described below) carbomer-based hydrogel (Carbopol®, BF Goodrich) was chosen as the preferred embodiment for the preparation of a fibronectin hydrogel. Preferred grade of polyacrylic acid is Carbopol 974-P at concentrations ranging from 0.281% to 0.375% (w/w) to which is added chlorocresol 0.1%, used as a bacteriostatic agent. Lower concentrations of carbomer are insufficient to promote adherence to the wound and higher concentrations were found to reduce the release of fibronectin from the hydrogel. Viscosity of carbomer hydrogels is stable between pH 6 to 8.

The hydrogel is prepared as follows: chlorocresol (1 g) is dissolved in warm deionized water (95 mL). Carbomer (3.75 g) is then dispersed into this solution and allowed to be mixed with a paddle type stirrer for about 3 hours. This dispersion is then autoclaved to provide a sterile concentrated hydrogel base (3.75% w/w). A stock solution of fibronectin containing 2.2 mg/mL (90 mL) is filtered through a 0.22 µm acetate filter. A polymerization promoter, sodium hydroxide, is then added to the fibronectin solution in an amount that will neutralize a 10 g portion of the carbomer 3.75% dispersion, that is 1250 µL of NaOH 3M. The stock solution of fibronectin and a 10 g portion of the carbomer dispersion are mixed into syringes taking care to avoid introducing air bubbles and contamination. This preparation provides a clear, preserved hydrogel (100 g) of fibronectin, free from microorganisms and with viscosity of about 410,000 cps.

1.1.3 Preparation of carbomer hydrogel containing fibronectin concentrations higher than 0.2%

Fibronectin was found to be highly insoluble when incorporated at concentrations higher that 0.2% in all formulations tested except carbomer hydrogels. We therefore prepared and tested the skin delivery capability of carbomer hydrogels containing up to 1% fibronectin. In order to prepare 10 g of fibronectin carbomer hydrogel containing higher concentrations of fibronectin, ranging from 0.3 to 1.0 %, the following ingredients must be added in sequence. First, the pH of 8.8 mL of demineralized water is adjusted at pH 8.0 to 11.0 with the addition of 2.95 µg to 2.95 mg NaOH 3M. The lyophilized fibronectin is next dissolved in demineralized water pH 8.0 to 11.0 in quantities varying from 3 mg to 10 mg. The solution is maintained

at 37°C until complete solubilization of fibronectin occurs and is filtered through a 0.22 µm acetate filter. In a final step of the procedure, 1 mL of water containing 0.028 g of carbopol and varying amounts of NaOH 3M, from 0.09399 g to 0.09105 g, is added to the mixture and mixed gently with syringes to produce a clear and air bubble-free fibronectin hydrogel. With formulations containing at least 0.3% fibronectin, it was observed that the carbomer concentration could be lowered to 0.281% (w/w) while maintaining the same wound adherence quality as a hydrogel containing 0.2% fibronectin and 0.375% carbomer. It was observed that the higher fibronectin content of the former formulation made up for the decreased wound adhesiveness that resulted from lowering the carbomer content.

1.2 Solid fibronectin calcium-alginate wound dressing formulation

Alginate salts can be converted into fibers by a process of freeze-drying. This procedure produces a sponge like structure with hydrophilic properties. In the presence of fluids, the dressing turns into a gel-like state, capable of absorbing up to 20 times its weight in wound exudate. The fibrous gel creates the desired moist environment for the wound. The dressing can be removed with a minimal amount of discomfort and granulating tissue and epithelial cells are not traumatized during dressing change. Calcium alginates dressings in particular are recommended for use on exudating wounds, such as pressure ulcers, venous stasis ulcers, diabetic ulcers, arterial ulcers, second degree burns and skin graft donor sites.

By combining the beneficial effect of calcium alginate and fibronectin, a calcium alginate dressing was developed with the ability to deliver a high concentration of fibronectin into the wound site. The basic mechanisms at play are that when the fibronectin-calcium alginate dressing comes into contact with the sodium in the exudate, ion exchange occurs, turning the calcium alginate fibers into a protective non-adherent film gel. In this gel state, fibronectin is free to move from the gel into the wound.

Solubilization

$$Ca\ (alginate)_2 + 2\ Na^+ \longrightarrow 2\ Na\ (alginate) + Ca^{++}$$

1.2.1 Fibronectin-calcium alginate dressing composition

The fibronectin-calcium alginate wound dressing is prepared as follows: 10 g of sodium alginate (Protanal LF 10/60, Pronova Biopolymer, Drammen, Norway) are dissolved in 90 g of deionized and demineralized water with a paddle type stirrer for about 1 hour. This dispersion is then autoclaved to provide a sterile concentrated alginate base (10 % w/w). The pH of 10 mL demineralized water is adjusted at pH 8.0 to 11.0 with the addtion of 3.35 µg to 3.35 mg NaOH 3M. The lyophilized fibronectin is next dissolved in demineralized water pH 8.0 to 11.0 in quantities varying from 0.025 to 0.1 g. The solution is maintaned at 37°C until complete solubilization of fibronectin occurs and is then filtered through a 0.22 µm acetate filter. The sterile solution of fibronectin (10 mL) is then mixed into syringes with a 1% solution sodium alginate (5 mL) in mild acetic acid pH 4.0 prepared from the concentrated alginate base. Gellation of the solution is achieved by the addition of 90 µL 0.2 M NaCl + 0.2 M $CaCl_2$ and 30 µL of glacial acetic acid. At this point, the fibronectin-calcium alginate complex is deposited in a plastic mold (5 mL for a surface area of 10 cm^2) and frozen. The water is then removed by freeze-drying. By this technique, a fibronectin/alginate wound dressing with a sponge-like structure is produced. This fibronectin-calcium alginate dressing is capable of absorbing 20 grams of saline solution (0.9% NaCl) per gram of dressing.

1.3 Fibronectin source: solvent/detergent-treated human homologous plasma

The source of fibronectin is human homologous plasma.

The risk of transmitting diseases by plasma products can be greatly diminished by taking two specific precautionary steps.

First, lots of plasma prepared from different donors are tested for atypical antibodies, hepatitis B and C virus (HBV, HCV), human immunodeficiency virus (HIV), human T-cell lymphotrophic virus (HTLV), cytomegalovirus (CMV) and syphilis.

Second, a viral inactivation solvent/detergent method using tri(n-butyl)phosphate (TNBP) and Triton X-100 is performed. Treatment of plasma products with organic solvent, tri(n-butyl)phosphate (TNBP) and Triton X-100 detergent was shown to

inactivate very large quantities of HBV, HCV and HIV (Horowitz et al., 1992) without affecting labile proteins such as fibronectin.

In a typical preparation, frozen plasma from 5 donors is thawed and treated while stirring for 6 hours with 1% (vol/vol) TNBP, 1% (vol/vol) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride at 24°C. After treatment, soybean oil (20% vol/vol) is added, mixed gently for 30 minutes at ambient temperature, and then removed by using a decantation funnel at 4°C.

Once fibronectin is purified from plasma (see section 1.3) the final solution is verified for contamination by TNBP and Triton X-100. TNBP is quantified in a sample of purified fibronectin after hexane extraction by gas chromatography using a 0.25-in by 2 mm ID by 4-ft glass column packed with 10% SP-1000 on a 80/100 mesh Supelcoport (Supelco, Bellafonte, PA). Triton X-100 was assayed by injecting a sample of purified fibronectin to high liquid chromatography (HPLC) on a gel filtration column G2000 SW 7.5 mm ID by 60 cm (Tosohass) coupled with a UV detector set at 230 nm. Fibronectin preparations were found to contain less that 1 ppm of either TNBP or Triton X-100.

1.3.1 Fibronectin purification from human homologous plasma by gelatin-Sepharose affinity chromatography

Fibronectin was isolated from solvent/detergent-treated human plasma using a gelatin-Sepharose affinity chromatography procedure (Horowitz and Chang, 1989). This method takes advantage of the affinity of fibronectin for gelatin in a procedure that allows isolation of electrophoretically pure fibronectin from human plasma with excellent yields.

In this method, gelatin is covalently coupled to Sepharose CL-4B after CNBr activation. The binding capacity for human plasma fibronectin provided by this system is > 1 mg/mL of gel. The purificaton is performed in a batch procedure with a glass funnel filter holder (Costar Nucleopore, Pleasanton, CA) with a capacity of 375 mL and a filtration area of 10.5 cm^2 at a flow rate of 25 mL/min.

Briefly, the plasma sample is passed twice on a gelatin-Sepharose gel. The matrix is washed with several volumes of 0.15 M Tris-HCl buffer pH 7.5, several volumes of

0.15 M Tris-HCl buffer pH 7.5 + 1 M NaCl and again with 0.15 M Tri-/HCl buffer pH 7.5. Elution is carried out with 1 M KBr in 0.1 M acetate buffer pH 5.0. The resulting solution of fibronectin is then exhaustively dialysed against deionized and demineralized water, ultrafiltered under nitrogen, lyophilized and frozen at -80°C until used.

The protein concentration is determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). The following diagram summarizes the purification steps.

Procedures for purifying fibronectin from human plasma treated with TNBP/Triton X-100

2.5 L of plasma (from 5 donors)

↓ 1.0% (v/v) TNBP + 1.0% (v/v) Triton X-100 6 hr/ 24°C

Solvent-Detergent plasma

↓ TNBP extraction with 20% (v/v) vegetable oil

Plasma + 200 mL of gelatin-Sepharose gel

↓

First wash Tris-HCl buffer 0.15 M pH 7.5

↓

Second wash Tris-HCl buffer 0.15 M + 1 M NaCl pH 7.5

↓

Third wash Tris-HCl buffer 0.15 M pH 7.5

↓

Elution sodium acetate buffer 0.1M + 1M KBr pH 5.0

↓

Dialysis against deionized and demineralized water

↓

Concentration by ultrafiltration under nitrogen

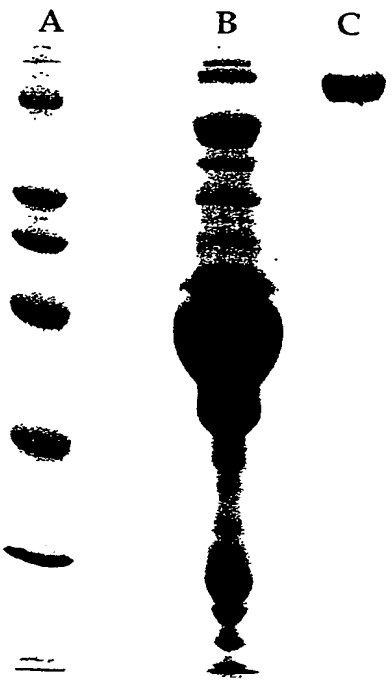
↓

Sterile filtration with 0.22 µm acetate filter

↓

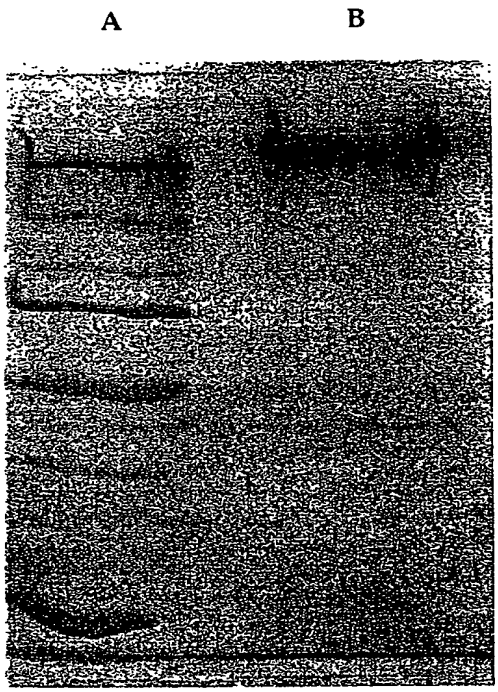
Lyophilization

In order to measure the level of purification of fibronectin isolated from human plasma, we performed sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1994) on gels formed with 7.5% acrylamide in 0.15 M Tris/HCl glycine buffer, pH 7.5, containing 0.1% SDS (w/v). Gels were stained with 0.18% Coomassie blue. Results showed a very high level of purification of fibronectin (Figure 1).



SDS-PAGE of purified fibronectin. Lane A: molecular weight standards 200 to 29 kDa; lane B: whole plasma proteins; lane C: freshly purified fibronectin. Protein bands were visualized with Coomassie brillant blue R250.

Figure 1A: Level of purification of fibronectin isolated from human plasma



SDS-PAGE of purified fibronectin. Lane A: molecular weight standards 200 to 29 kDa; lane B: freshly purified fibronectin. Proteins bands were visualized using the color-based silver stain method.

Figure 1B: Level of purification of fibronectin isolated from human plasma

2. PHARMACOKINETICS AND BIOLOGICAL DISPOSITION OF THE DRUG IN HUMANS

2.1 In vitro study of absorption of fibronectin in deepithelialized human skin using a cell diffusion system

The effectiveness of different topical formulations to deliver fibronectin in skin was evaluated using an *in vitro* diffusion cell system and deepithelialized human skin. In skin ulcers, the barrier effect of the stratum corneum is absent and only the diffusion from the dermatological vehicle will constitute a determinant barrier for the effective penetration of the drug into the dermis. Therefore, absorption studies were all performed on human breast and abdominal deepithelialized human skin samples obtained from breast reduction and abdominal lipectomy surgeries. For deepithelialization, a 8 μm section was removed from the epidermal surface of the

skin using a dermatome (1/10 000 scissor scale) and dermal side was carefully cleaned of any adhering subcutaneous tissues and/or blood vessels.

The diffusion cell system selected consists of a rigid receptor containing the skin sample, the deepithelialized side facing upwards into the donor compartment and the dermal side facing downwards into the receptor compartment. The receptor compartment is connected to a circulating buffer circuit. The buffer temperature is maintained at 37°C while the skin surface is at about 32°C. Each analysis is performed on a 0.64 cm^2 skin sample using a 100 mL aliquot of ^{125}I-fibronectin (approximately 10^6 cpm) in the topical formulation. The diffusion rate of fibronectin from different topical preparations across deepithelialized human skin from plastic surgery is investigated using Teflon® Flow-Thru® Diffusion Cells (Crown Glass Co. Inc. Somerville, NJ) which have a surface area of 0.64 cm^2 (Bronaugh and Stewart, 1985). The diffusion cells are designed such that fluid may be continuously pumped through them in order to maintain sink conditions. A PBS buffer having a pH of 7.2 is used as perfusion fluid. The diffusion cells are mounted in a PosiBloc® Diffusion Cell Heater (Crown Glass Co. Inc. Somerville, NJ) maintained at 32°C by a circulating water bath. The flow rate is 3 mL per hour. Each experiment is conducted for a continuous period of 24 hours. After the experiment, the skin is removed from the diffusion cell, washed 10 times with 8 mL of water for each wash. The final wash fluid contains less than 0.2% of the initial amount of radioactivity added to each cell. The total amount absorbed is calculated using the following formula:

$$\%\text{Fibronectin absorbed in skin} = \frac{\text{Total cpm applied} - [\text{Total cpm in total wash fluid} + \text{Total cpm diffused in lower cell}]}{\text{Total cpm applied}} \times 100$$

In experiments using hydrogels containing concentrations of fibronectin higher than 0.2%, an ELISA assay is used to measure skin absorption instead of ^{125}Iodine-labelled protein. A polystyrene microtiter plate (NUNC) is coated with 100 µL of known concentrations of fibronectin from 0.1 to 2.0 µg/100 µL in 50 mM carbonate buffer, pH 9.6 at 4°C overnight. The plate is next washed three times with PBS-Tween buffer and unoccupied binding sites are blocked with 5.0% of BSA in PBS-Tween buffer at 37°C for 1 hour. After rinsing with PBS-Tween buffer, 100 µL of polyclonal antibody to human fibronectin are added and the plate is incubated 1 hour at 37°C. After rinsing three times with PBS-Tween buffer, 100 µL of goat anti-mouse IgG antibody horseradish peroxidase conjugated (IgG-HRP) (diluted 2000-fold with 0.5% BSA

diluted in PBS-Tween buffer) are added and the plate is incubated 1 hour at 37°C. Excess conjugate is then thoroughly removed by washing, and peroxidase fixed to the wells is detected by addition of ABTS and peroxide substrate diluted in sodium citrate buffer pH 4.6. In order to determine the amount of fibronectin absorbed in the skin, ELISA measurement are performed in the total wash fluid as well as in the fluid that had accumulated in the inner compartment of the cell diffusion system. The reaction is followed by increases in absorbance at 410 nm and comparison with a standard peroxidase reaction. The amount of fibronectin absorbed in deepithelialized human skin is determined using the following formula:

$$\%\text{Fibronectin absorbed in skin} = \frac{\text{Amount applied}(\mu g) - [\text{Amount in total wash fluid}(\mu g) + \text{Amount diffused in lower cell}(\mu g)}{\text{Amount applied}(\mu g)} \times 100$$

2.2 Kinetics of release of fibronectin from different pharmaceutically acceptable topical formulations

The precise composition of the different formulations tested is presented in Annex A. These were chosen based on their pharmaceutically acceptable dosage forms to treat clinical skin conditions. All dosage forms were made in salt-free solution since viscosity values could have been influenced by the presence of electrolytes. For instance, viscosity values of carbomer hydrogels are reduced in presence of strong electrolytes. This is in contrast to poloxamer hydrogels which are more viscous when electrolytes are added to the preparation. The maximal concentration of fibronectin in the dosage form was 0.2% in the results of experiments presented in view of the fact that in all preparations, except carbomer hydrogels, fibronectin is found to be poorly soluble at higher concentrations, especially under conditions of storage of the hydrogel at 4°C.

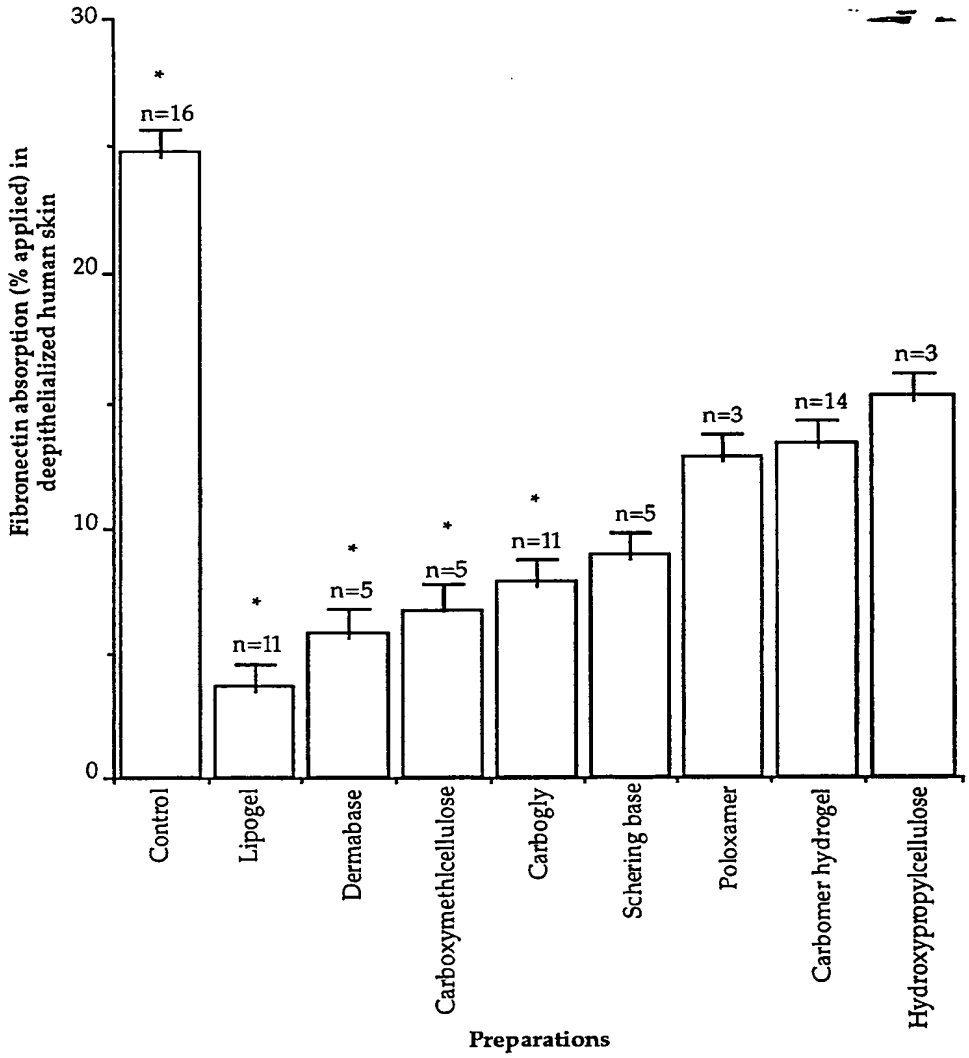
Kinetic data of the release of fibronectin from dosage forms were obtained at 4, 12, and 24 hours. Table 1 summarizes these data for t=12 hours. Control consisted of ^{125}I-fibronectin in PBS solution pH 7.4.

Table 1. Absorption in deepithelialized human skin of fibronectin embodied in different topical pharmaceutical formulations

Formulation	[ ]	Abs value (%)
Control	PBS	24.75
Carbomer Hydrogel + Liposomes 3090 SH® (Lipogel)	1%/15%	3.70
Dermabase®	(1:1)	5.80
Carboxymethylcellulose gel	3%	6.70
Carbomer Hydrogel + glycerol (Carbogly)	0.375%/10%	7.80
Schering base®	(1:1)	9.90
Poloxamer	20%	12.80
Carbomer Hydrogel	0.375%	13.40
Hydroxypropylcellulose gel	3%	15.20

Precise composition of each formulation is described in Annex A. The symbol [ ] refers to components concentration. "Abs value" refers to the percentage of radiolabelled fibronectin measured in the dermis. Absorption time = 12 hours. Control = phosphate buffered saline.

In Figure 2, the same data is presented. However, the Figure also illustrates the outcome of the statistical analysis of the findings. Of note are the low standard deviations, showing excellent performance of the deepithelialized human skin cell

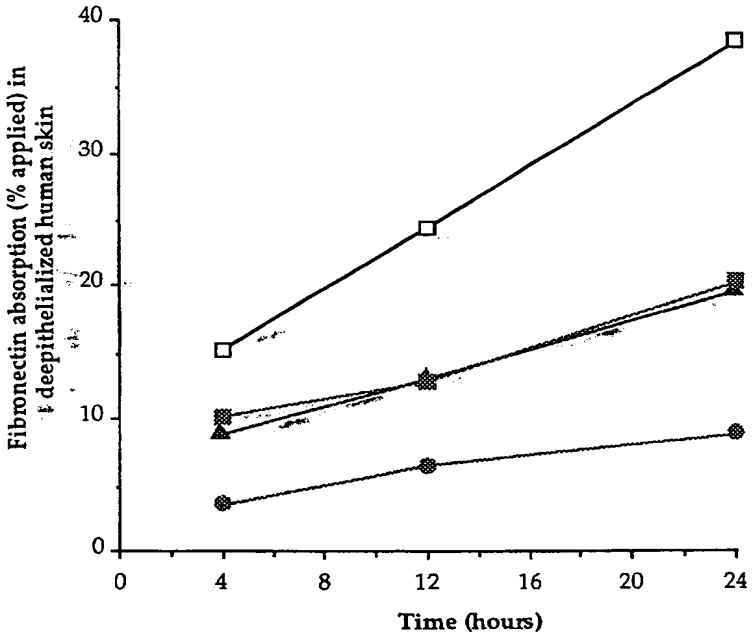


Deepithelialized human skin absorption of radiolabelled ^{125}I-fibronectin using different topical pharmaceutical formulations. The precise composition of all pharmaceutical formulations are described in detail in Annex A. The Dunnett statistical test was used to identify statistically significant differences between carbomer hydrogel and other formulations. Bars represent standard deviations of the mean.* Statistically different from Carbomer Hydrogel (P<0,001).

Figure 2: Absorption of ^{125}I-labelled fibronectin in deepithelialized human skin using different topical pharmaceutical formulations

diffusion system in inter-experimental variations. Marked differences in absorption values are observed between certain preparations, while others are similar. Among preparations yielding equivalent amounts of fibronectin in deepithelialized human skin (poloxamer, carbomer hydrogel and hydroxypropylcellulose), only the carbomer hydrogel formulation was considered an appropirate carrier for the following reasons: 1- hydroxypropylcellulose hydrogel has very poor wound adherence capability; 2- in poloxamer and hydroxypropylcellulose hydrogels, fibronectin is poorly soluble above a 0.2% concentration.

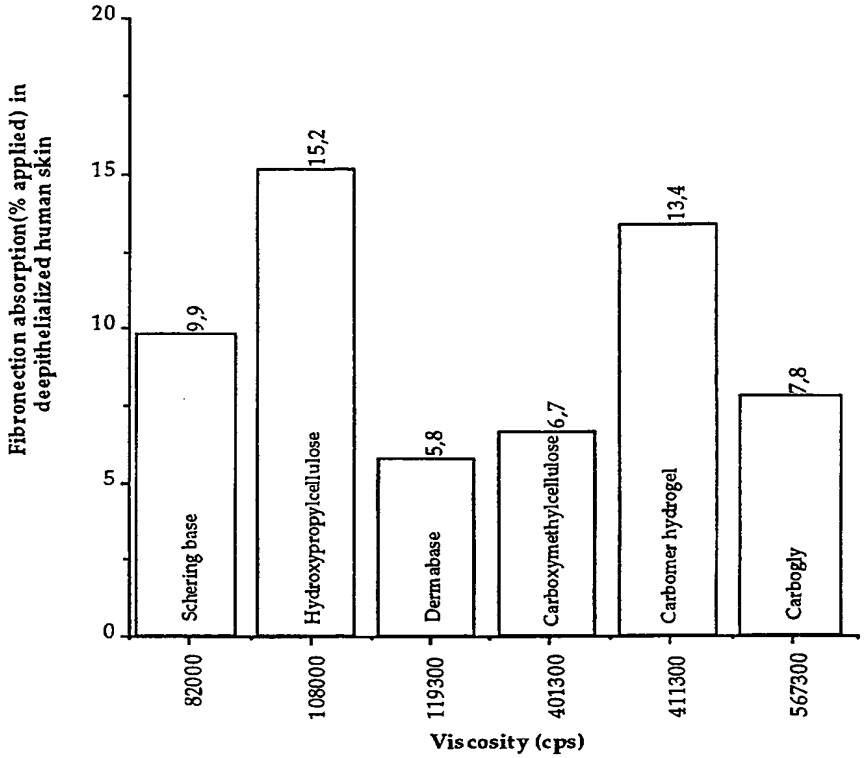
In Figure 3, data of the cumulative absorption of ^{125}I-labelled fibronectin over time from various hydrogel formulations is presented. Results are compared to the control (phosphate buffered saline solution). From this Figure, it can be seen that the absorption process tends to be more important between 0 and 12 hours when compared to that occurring between 12 and 24 hours, suggesting that two applications per day could release more fibronectin than a once a day schedule.



Cumulative absorption of ^{125}I-labelled fibronectin in deepithelialized human skin over time comparing different pharmaceutical topical formulations: (□) Control (phosphate buffered saline solution); (▲) Carbomer hydrogel (0.375%); (▩) Poloxamer (20%); and (●) Carboxymethylcellulose (3%).

Figure 3: Cumulative absorption over time in deepithelialized human skin of ^{125}I-labelled fibronectin from different topical pharmaceutical formulations

Figure 4 demonstrates that viscosity and level of absorption are not related. For instance, Dermabase® cream base has a relatively low viscosity (119,000 cps) when compared to carbomer hydrogel (411,000 cps) and presents poor release capabilities (5.80%) when compared to this carbomer hydrogel (13.40%). The efficacity of the carbomer formulation is particularly surprising since carbomer has a higher degree of viscosity than many of the other formulations studied. Fibronectin is a very high molecular weight protein and it could have been expected that the high viscosity of carbomer hydrogel would have constituted a poor carrier for effective release of fibronectin into the skin. Also noteworthy is the difference in absorption values between carbomer and carboxymethylcellulose formulations since they share the same degree of viscosity.



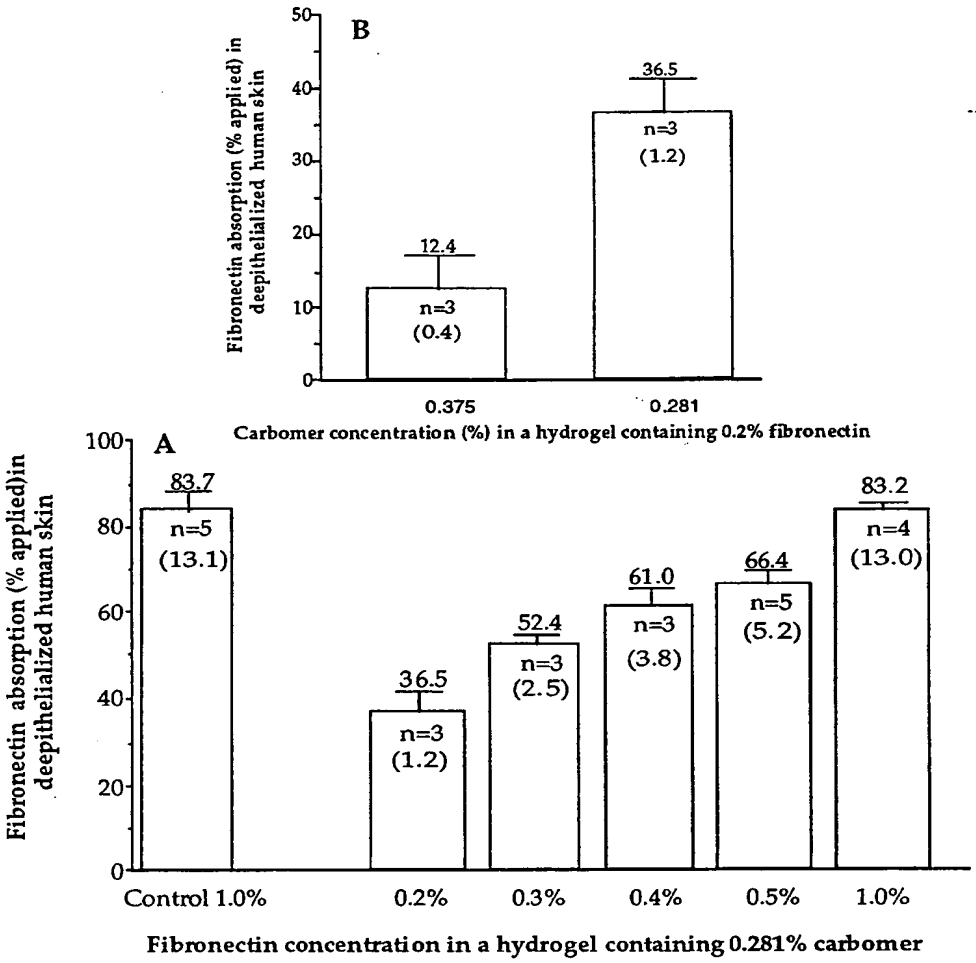
Relationship between viscosity of topical formulations and deepithelialized human skin absorption of fibronectin. All viscosity values are in centipoises (cps) as measured with a Brookfield viscometer. Assays were performed at 0.5 rpm and at room temperature.

Figure 4: Deepithelialized human skin absorption of ^{125}I-labelled fibronectin versus viscosity of topical pharmaceutical formulations

2.3 Effect of fibronectin and carbomer concentrations in the hydrogel on the absorption of fibronectin in deepithelialized human skin

The release of fibronectin from formulations containing higher concentrations of fibronectin was evaluated *in vitro* with the cell diffusion system described above. The amount of fibronectin in the skin was assayed by ELISA instead of ^{125}I-labelled fibronectin. The amount of fibronectin released into the skin was determined as described in section 2.1.

Results are shown in Figure 5 A and reveal that as much as 13 µg of fibronectin per mm^2 can be delivered in deepithelialized human skin over a 12 hour period. This amount is comparable to a 1% solution of fibronectin in phosphate buffered saline. Furthermore, it was observed that lowering the carbomer concentration of the hydrogel was a determining factor affecting the release of fibronectin into skin. In Figure 5 B is shown the effect of carbomer concentration on the absorption of fibronectin in deepithelialized human skin. For a same concentration of 0.2% of fibronectin, but for two different carbomer concentrations (0.281% and 0.375%), the amount of fibronectin in the skin increases significantly from 12.4% to 36.5%. This clearly demonstrates the effect of carbomer concentration on the permeability of the hydrogel for fibronectin absorption in deepithelialized human skin.

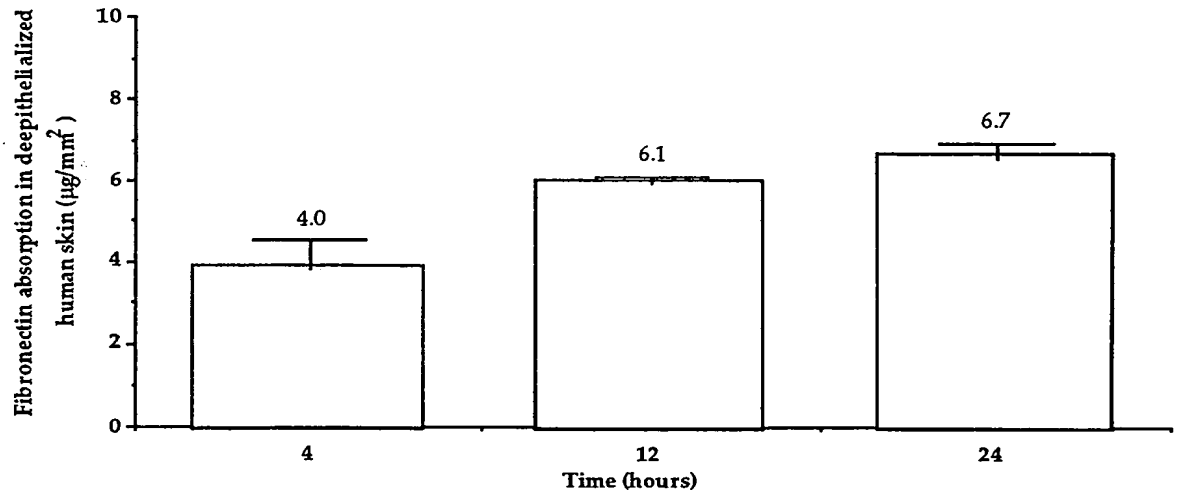


A.Effect of varying the concentration of fibronectin (0.2% to 1.0%) in a 0.281% carbomer hydrogel on fibronectin absorption in deepithelialized human skin. Control is a 1.0% fibronectin phosphate buffered saline solution. B. Effect of varying the carbomer concentration (0.281% and 0.375%) on the absorption of fibronectin in deepithelialized human skin using a 0.2% fibronectin carbomer hydrogel. The number in ( ) refers to the quantity of delivered fibronectin (μg) per mm^2 of deepithelialized human skin over a 12 hour period. Bars represent standard deviations of the mean.

Figure 5: Effect of the concentrations of fibronectin and carbomer on the absorption of fibronectin in deepithelialized human skin after 12 hours of absorption

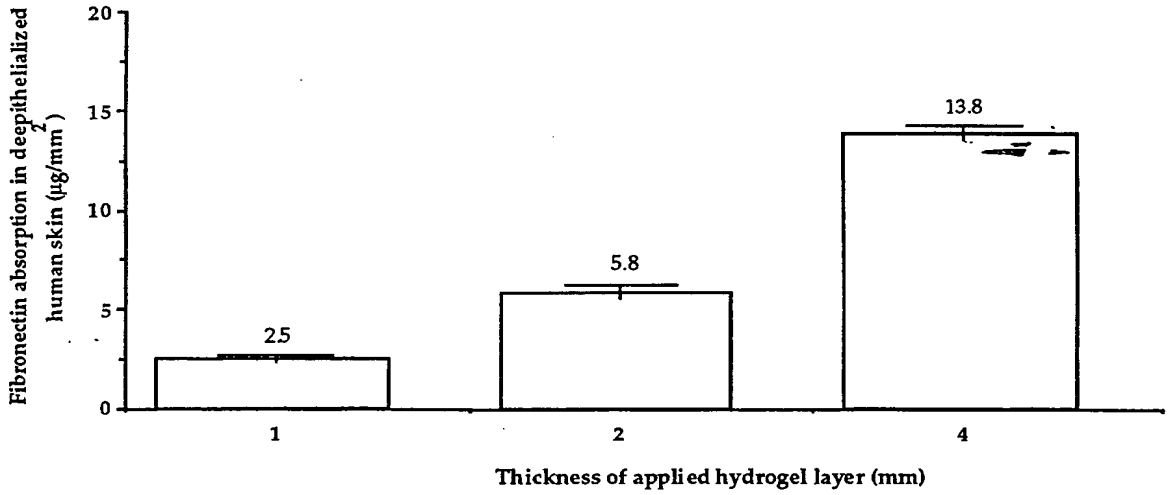
In Figure 6 data of the cumulative absorption of fibronectin over time from a 0.5% fibronectin hydrogel is presented. In agreement with the previous obtained results from the Figure 3 with another fibronectin hydrogel formulation, it can be seen that the absorption process tends to be more important between 0 and 12 hours. Up to 6.1 $\mu g/mm^2$ of fibronectin are absorbed in deepithelialized human skin in 12 hours whereas only 6.7 $\mu g/mm^2$ are absorbed in 24 hours, suggesting that two applications per day could release more fibronectin than a once a day schedule. It is established that there is a greater chance of healing when the ulcer is the least disturbed.

In Figure 7 is shown the effect of the thickness of applied 0.5% fibronectin carbomer hydrogel on the absorption of fibronectin in deepithelialized human skin. For a twelve hour period of absorption, by varying the thickness from 1 to 4 mm, the amount of fibronectin in the skin increases significantly from 2.5 to 13.8 $\mu g/mm^2$, suggesting that a once a day schedule could be beneficial with an increase of the applied volume.



Cumulative absorption of fibronectin in deepithelialized human skin over time in a 0.281% carbomer hydrogel. Bars represent standard deviations of the mean (n=3).

Figure 6: Cumulative absorption over time in deepithelialized human skin of 0.5% fibronectin carbomer hydrogel



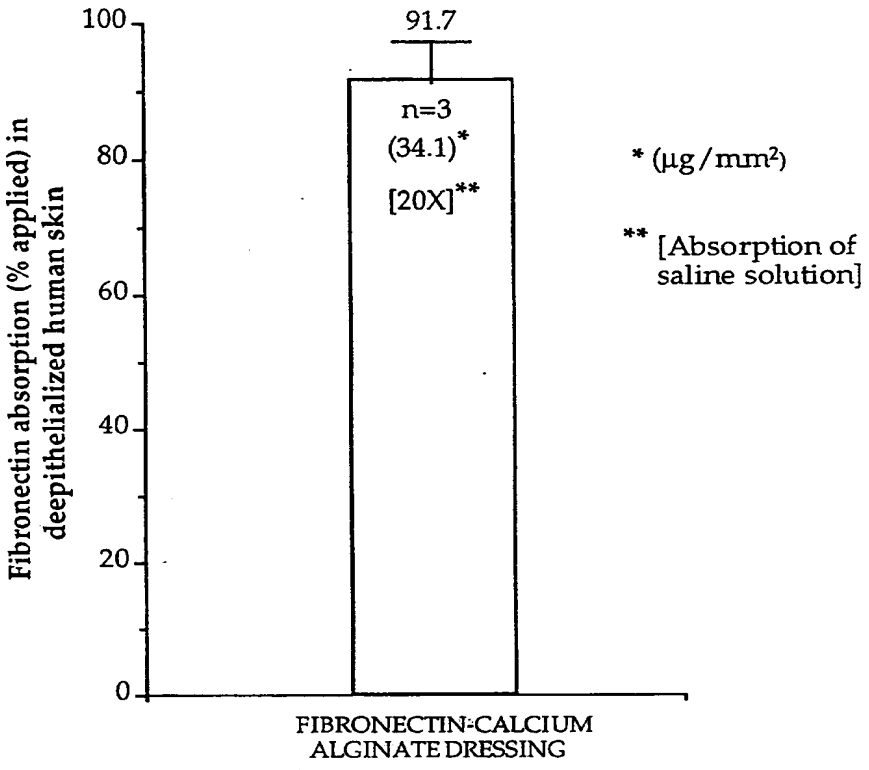
Effect of varying the amount of applied fibronectin in a 0.281% carbomer hydrogel on fibronectin absorption in deepithelialized human skin over a 12 hour period. Bars represent standard deviations of the mean (n=3).

Figure 7: Effect of varying the amount of 0.5% fibronectin carbomer hydrogel applied to deepithelialized human skin

2.4 Kinetics of release of fibronectin from fibronectin-calcium alginate solid dressing

The amount of fibronectin released into the skin from the fibronectin-calcium alginate dressing was determined using a cell diffusion system as described in section 2.1. Each analysis was performed on a 0.64 cm^2 deepithelialized human skin sample. From a solid fibronectin-calcium alginate dressing of 9.1 cm^2 surface area containing 64% (w/w) fibronectin, a piece measuring 9-mm in diameter, and corresponding to 0.64 cm^2 surface area, was cut using a 9-mm trephine. It was then weighed and placed on the surface of the skin. The mean amount of fibronectin applied using the fibronectin-calcium alginate dressing was 2.3 mg. In order to mimic exudate being produced by the wound, the solid dressing was wetted with 100 µL of saline solution. Each experiment was conducted for a continuous period of 24 hours. In order to determine the amount of fibronectin absorbed in the deepithelialized human skin, ELISA measurements were performed as described in section 2.1.

In vitro study of the absorption of fibronectin in deepithelialized human skin using a cell diffusion system showed that up to 91.7% of the fibronectin was released from the fibronectin-calcium alginate dressing after a 24 hour period (Figure 8). This amount represents 34.1 µg of fibronectin delivered per mm^2 of deepithelialized skin surface area.



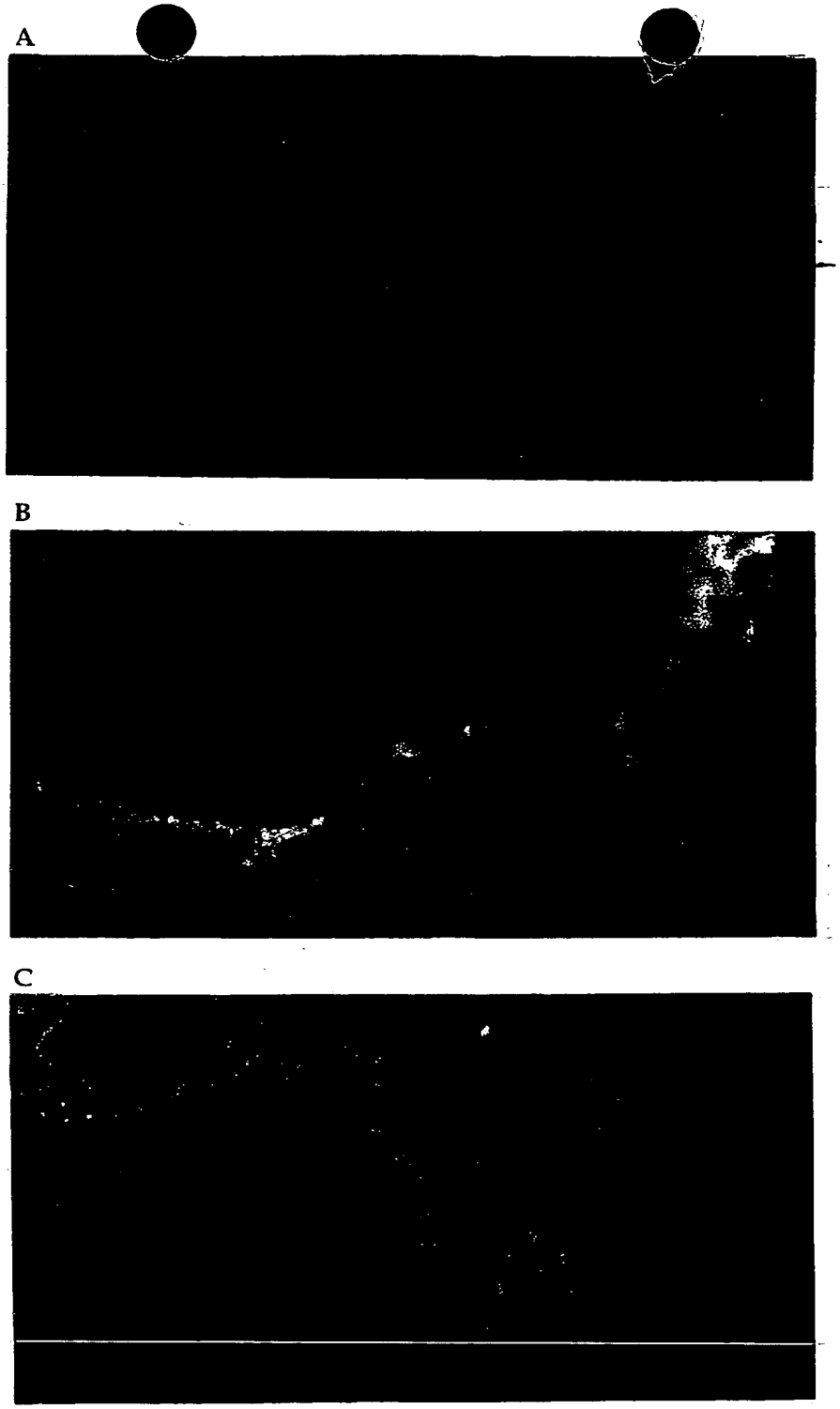
Absorption of fibronectin in deepithelialized human skin using different solid wound dressings. The number in ( ) refers to the quantity of absorbed fibronectin (µg) per mm^2 of deepithelialized human skin over a 12 hour period. The number in [ ] refers to the quantity of absorbed saline solution (0.9% NaCl) by weight of dressing. Bar represents standard deviation of the mean.

Figure 8: Absorption of fibronectin in deepithelialized human skin using the fibronectin-calcium alginate solid wound dressings over a 24 hour period

2.5 Immunofluorescence labelling of tissue sections to study fibronectin distribution and depth of penetration in deepithelialized human skin

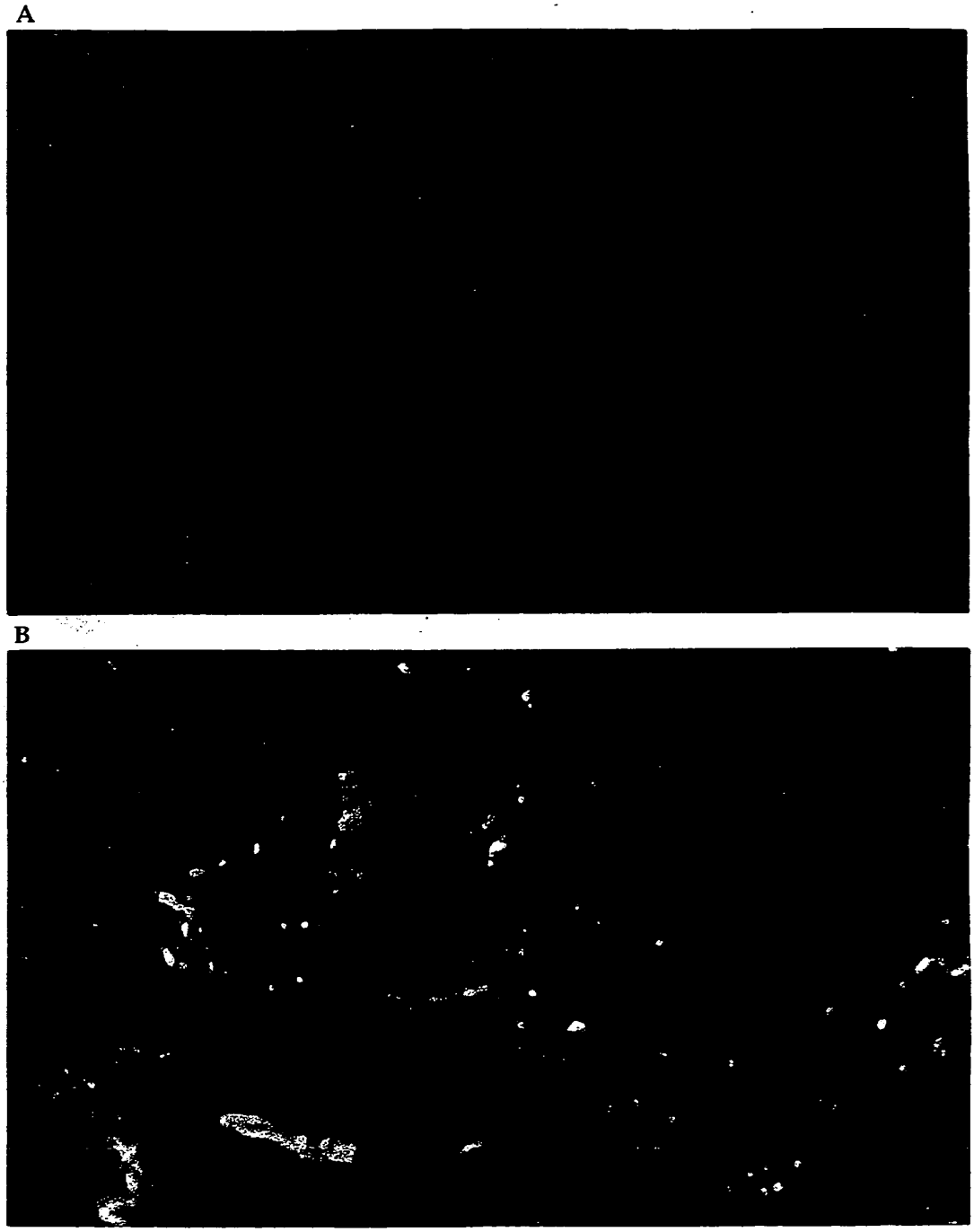
In order to visualize the distribution and penetration of fibronectin in a 3 mm thick (3000 µm) deepithelialized human skin sample after 12 hours of application of a carbomer hydrogel containing 0.5% of fibronectin, we performed, at different skin depths, immunofluorescence staining on frozen skin sections of 2 µm thickness using an antibody to human fibronectin. Sections were first incubated with a mouse monoclonal antibody to human fibronectin (Sigma, MO) that was diluted 1/200 in PBS. The second antibody was a goat-anti mouse Ig that was FITC conjugated (Sigma, MO) and diluted 1/50. Both reactions were conducted for 30 minutes at room temperature with washes in PBS between reactions. Coverslips on which were placed skin samples were finally mounted in an aqueous mounting medium containing phosphate buffered glycerol, and analyzed under a Leitz Photo microscope equiped with epifluorescence optics.

Figure 9 shows the distribution of fibronectin in a 2 µm frozen skin section as detected by indirect immunofluorescence at a magnification of 400 X whereas Figure 10 shows the distribution of fibronectin at a magnification of 100 X. The results are in agreement with the previous data obtained using ELISA, and show relatively diffuse and deep penetration of fibronectin in the skin.



A carbomer hydrogel containing either no fibronectin (negative control) or 0.5% fibronectin (positive control) was applied on deepithelialized human skin for 12 hours. Frozen sections were 2 µm thick. A: negative control at 8 µm depth. B: positive control at 482 µm depth. C: positive control at 2 000 µm depth. Total tickness of skin sample was 3000 µm. Magnification 400 X.

Figure 9: Immunofluorescence detection of fibronectin in deepithelialized human skin



A carbomer hydrogel containing either no fibronectin (negative control) or 0.5% fibronectin (positive control) was applied on deepithelialized skin for 12 hours. Frozen sections were 2 µm thick. A: negative control at 417 µm depth. B: positive control at 435 µm depth. Total tickness of skin sample was 3000 µm. Magnification 100 X.

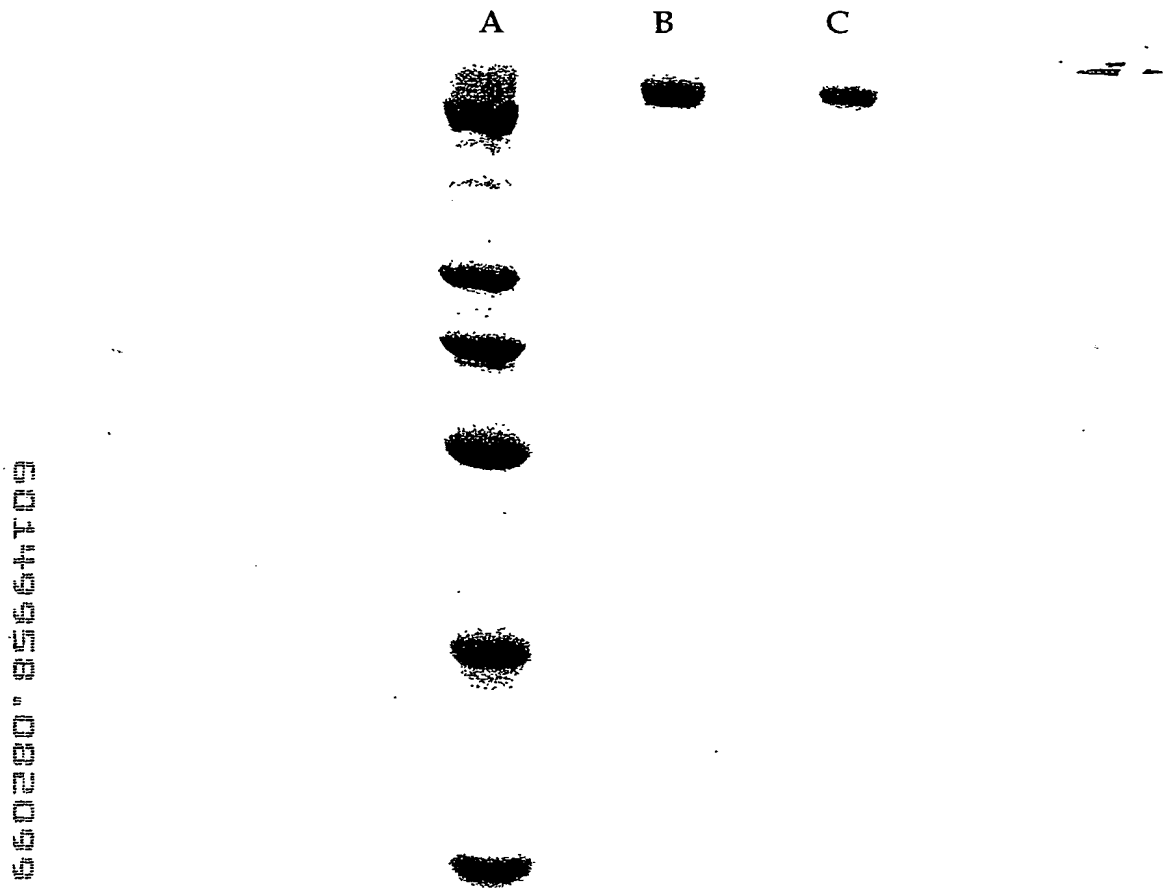
Figure 10: Immunofluorescence detection of fibronectin in deepithelialized human skin

3. STRUCTURAL AND FUNCTIONAL STABILITY OF FIBRONECTIN IN A 0.2% FIBRONECTIN HYDROGEL STORED AT 4°C DURING 12 MONTHS

The structural and functional stability of fibronectin in the carbomer hydrogel was analyzed. Assays were performed on a specimen of hydrogel containing 0.2% fibronectin in a 0.375% carbomer hydrogel. The hydrogel was kept at 4°C for 12 months.

3.1 Structural stability of fibronectin in carbomer hydrogel

Electrophoresis on SDS-PAGE was performed in order to determine the structural stability of fibronectin in the hydrogel. Once the specimen of hydrogel was dissolved in NaCl 1 M + Tris-HCl pH 7.4 solution, it was allowed to migrate on a 7.5% polyacrylamide gel, according to the method described by Laemmli. When compared to a fresh preparation of fibronectin, results showed that essentially all of the fibronectin migrated at the 220,000 molecular weight marker, indicating that little, if any, degradation had occurred.



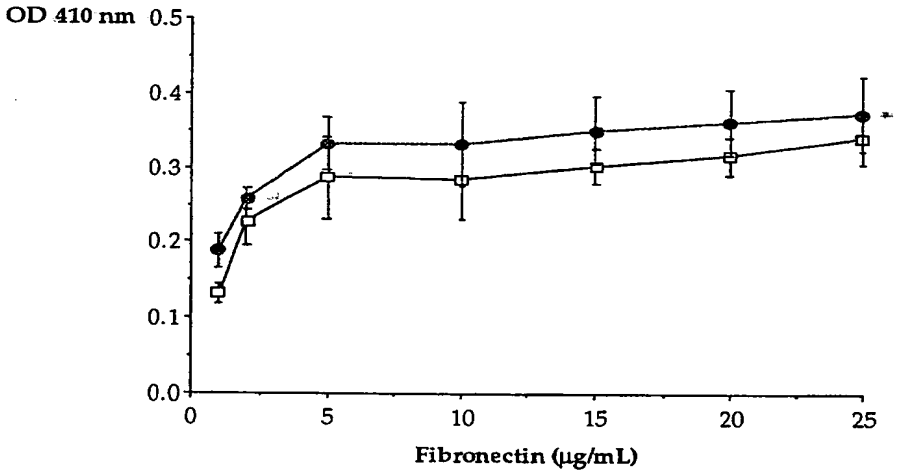
SDS-PAGE of different fibronectin preparations. Lane **A**: molecular weight standards 200 to 29 kDa. Lane **B**: fresh fibronectin preparation. Lane **C**: fibronectin extracted from a hydrogel specimen stored 12 months at 4°C. Protein bands were visualized with Coomassie brillant blue R250.

Figure 11: Structural stability of fibronectin in the carbomer hydrogel

3.2 Functional stability of fibronectin in carbomer hydrogel

3.2.1 Gelatin-binding capacity

Gelatin-binding capacity is an intrinsic function of fibronectin. Gelatin-binding capacity was assayed using the method described by Regnault et al. (1988). Dilutions and washes were carried out in PBS-Tween buffer pH 7.2. Ninety-six well polystyrene plates were coated with gelatin (100 µL of a stock solution at 5 mg/mL in 50 mM sodium carbonate buffer pH 9.6) overnight at room temperature. After washing with PBS-Tween, wells were filled with 100 µL of different dilutions of either freshly prepared fibronectin or fibronectin extracted from the hydrogel (1 to 25 µg/mL) and incubated 30 min at 37°C. After washing, 100 µL of rabbit polyclonal antibody to human fibronectin was diluted 1/100 000 and were added to each well. The plate was incubated 1 hour at 37°C. After washing, 100 µL of peroxidase labelled goat antibody to rabbit IgG and IgM diluted 1/ 50 000 were added and the plate was again incubated for 1 hour at 37°C. Finally, after washing, enzymatic activity was measured by the addition of 100 µL of a solution containing peroxide substrate and 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) in sodium citrate buffer pH 4.6. Optical density was measured at 410 nm. The gelatin-binding activity of fibronectin extracted from the carbomer hydrogel stored 12 months at 4°C was found to be similar to that of freshly purified fibronectin (P = 0.1913).

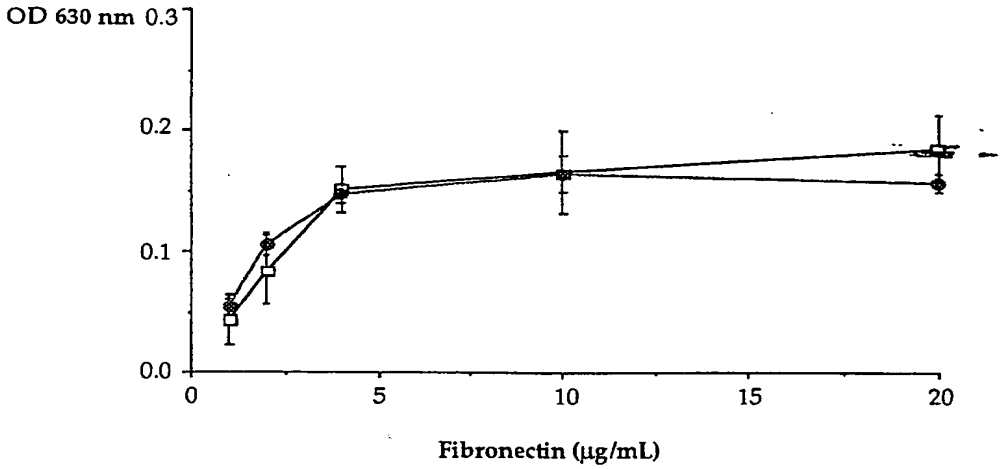


Polystyrene microtiter plate was coated with gelatin and incubated with different dilutions of: (□) fresh preparation of fibronectin; (●) fibronectin extracted from carbomer hydrogel. Rabbit polyclonal antibody to human fibronectin and peroxidase labelled goat antibody to rabbit IgG and IgM were then respectively added to the wells. n = 4; p = 0.1913 (Student's two-tailed t tests).

Figure 12: Gelatin-binding activity of fibronectin extracted from the carbomer hydrogel stored at 4°C during 12 months

3.2.2 Cell adhesion promoting activity

The promotion of cell adherence is another intrinsic property of fibronectin. This assay was performed according to the method described by Ruoslahti et al. (1982). Human virus-transformed skin fibroblasts GM00637E were used. One day before the assay, a confluent culture of cells was split 1:2. For the assay, the cells were washed three times with PBS buffer pH 7.5 and incubated with 10 mL of trypsin (Sigma, type III) at 0.1 mg/mL in PBS; cells were incubated at 37°C until they detached. The cells were then collected by centrifugation and washed three times with a solution of 0.5 mg/mL soybean trypsin inhibitor in PBS for neutralization of trypsin. Before the last centrifugation, a sample was taken and cell number and viability by trypan blue exclusion were determined. The cells were then suspended in DMEM (Difco Laboratories) at a concentration of 2 X 10^6 cells/mL and dispersed by pipetting until a single-cell suspension was obtained. Ninety-six well polystyrene plates were coated with 100 µL of 5 mg/mL of gelatin in 50 mM sodium carbonate buffer pH 9.6. Fibronectin fixation to gelatin was performed with 100 µL of varying concentrations of fibronectin (1 to 20 µg/mL) in 50 mM of sodium carbonate buffer, pH 9.6, for a period of 2 hours at room temperature. Unbound protein was removed by washing three times with PBS. To ensure even dispersal of the cells in the microtiter well, 100 µL of DMEM were added to each well. This was followed by the addition of 100 µL of cell suspension. The plate was incubated for 1 hr at 37°C in 5 % CO_2. After 1 hour, the non-attached cells were removed simply by pouring out the medium and washing. The plate was flooded with PBS, and the washing solution was poured out. The cells were then fixed with 3% paraformaldehyde in PBS and stained with 1% toluidine blue, 3% paraformaldehyde in PBS. Attached cells were measured by absorbance at 630 nm. The cell adhesion promoting activity of fibronectin extracted form the carbomer hydrogel stored 12 months at 4°C was found to be similar to that of freshly purified fibronectin (P = 0.2975, Figure 13).



Polystyrene microtiter plate coated with gelatin is incubated with different dilutions of: (□) fresh preparation of fibronectin or (●) fibronectin extracted from hydrogel. GM00637E cells are next added to the wells for a period of 1 hour at 37°C. The cells are then fixed with paraformaldehyde and stained with toluidine blue. Attached cells were measured by absorbance at 630 nm. n = 3; p = 0.2975 (Student's two-tailed t tests).

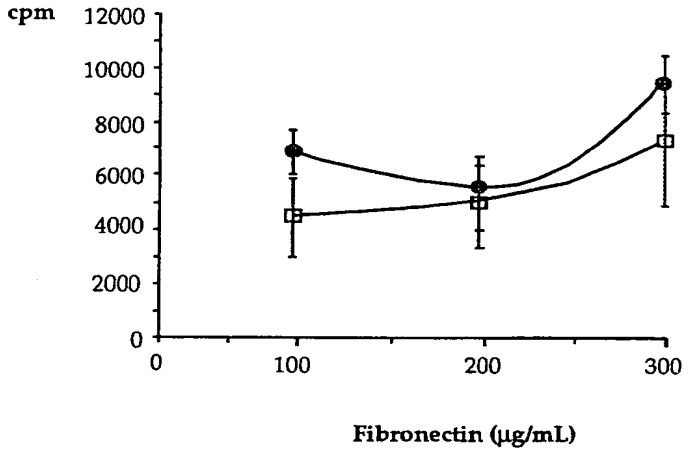
Figure 13: Cell adhesion promoting activity of fibronectin extracted from the carbomer hydrogel stored at 4°C during 12 months

3.2.3 Opsonin activity

The capacity of fibronectin to promote the phagocytosis (opsonin activity) of gelatin-coated beads is well documented. This property of fibronectin was assayed using a procedure described by Regnault et al. (1988) with the exception that human neutrophils were used instead of P 388 D1 murine cells. This method measures the phagocytosis of iodinated gelatin-latex beads by phagocytic cells. Coupling of gelatin to carboxylated latex beads was performed according to Molnar et al. (1987). Briefly, 5 mL (10% w/v) carboxylated latex (Bacto-latex 0,81 Difco), 5 mL 1% gelatin in PBS pH 7.4, and 0.5 mL of dicyclohexylcarbodiimide (10% w/v in dimethylformamide) were mixed and incubated at room temperature for 3 h, and at 4°C overnight. The beads were sedimented by centrifugation at 10,000 rpm for 20 min, resuspended in 40 mL of PBS pH 7.4 containing 1 mg/mL of bovine serum albumin and centrifuged again. The sediment was washed with PBS pH 7.4 two more times and then suspended in 10 mL of PBS pH 7.4. Radiolabelling was performed according to the method described by Molnar et al (1987). After washing the beads, the suspension was mixed with 400 µCi of $Na^{125}I$ (carrier free) and 200 µL of chloramine T (0.1% in PBS pH 7.4) and then incubated at 37°C for 20 min. The reaction was stopped by adding 200 µL of

0.5% sodium tetrathionate and the labelled particles were washed 5 times with 40 mL PBS pH 7.4 and centrifuged. Finally, the pellet was suspended in 10 mL of PBS pH 7.4, and dialyzed against four changes of 4 L PBS pH 7.4 containing 0.1% NaI at 4°C. The particles were sedimented by centrifugation and the pellet was resuspended in 20 mL PBS pH 7.4 containing 1 mM EDTA, 1 mM PMSF and 0.1% NaN_3.

Iodinated gelatin-latex beads (50 µL) were added to a mixture of heparin (50 µL of 100 U/mL stock) and 100 µL of a solution containing different concentrations of fibronectin (100 to 300 µg/mL). The mixture was preincubated at 37°C, usually for two minutes, in a 12 X 75 mm polystyrene tube. Human neutrophils (250 µL of a suspension containing about 2 X 10^7 cells/mL) were then added and the incubation was continued for one hour at 37°C. At intervals, aliquots (100 µL) were transferred to tubes containing 2 mL of ice-cold PBS with divalent cations and 1 mM N-ethylmaleimide. Samples were then washed two times by rapid centrifugation (700 g for 10 seconds) and cells were counted for ^{125}Iodine in a gamma counter. The opsonin promoting activity of fibronectin from the carbomer hydrogel stored 12 months at 4°C was found to be similar to that of freshly purified fibronectin (P = 0.5464, Figure 14).



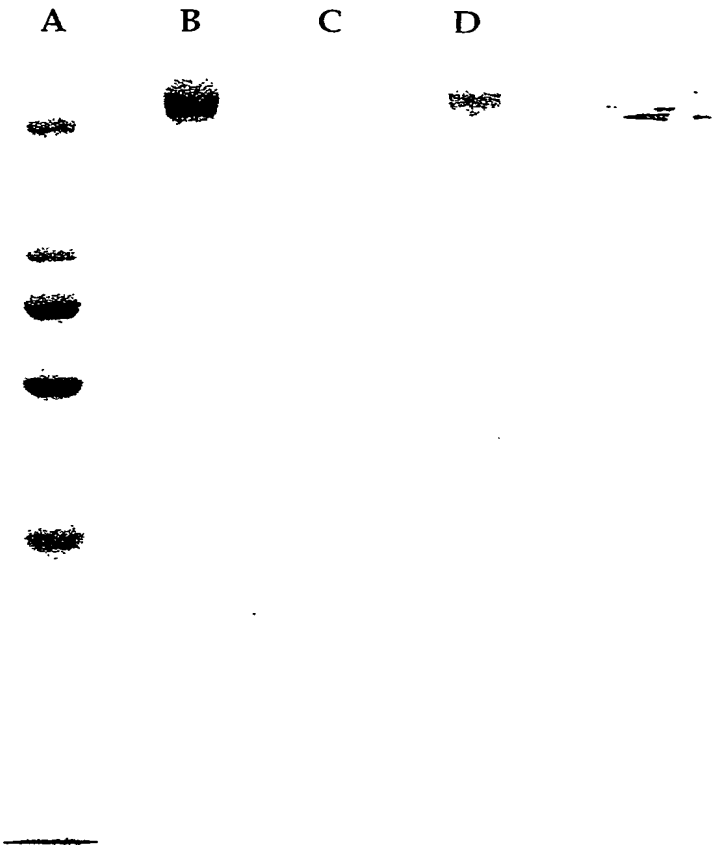
Human neutrophils were incubated with iodinated gelatin-latex beads and with (□) fresh preparation of fibronectin or (●) fibronectin extracted from carbomer hydrogel. Beads phagocytized by neutrophils are separated from free iodinated-latex beads with two rapid centrifugations. The radioactivity associated with neutrophils was counted for ^{125}Iodine in a gamma counter. n = 3; p = 0.5464 (Student's two-tailed t tests).

Figure 14: Opsonin activity of fibronectin extracted from the carbomer hydrogel stored at 4°C during 12 months

3.3 Structural and functional stability of fibronectin extracted from a carbomer hydrogel prepared with concentrations of fibronectin higher than 0.2%

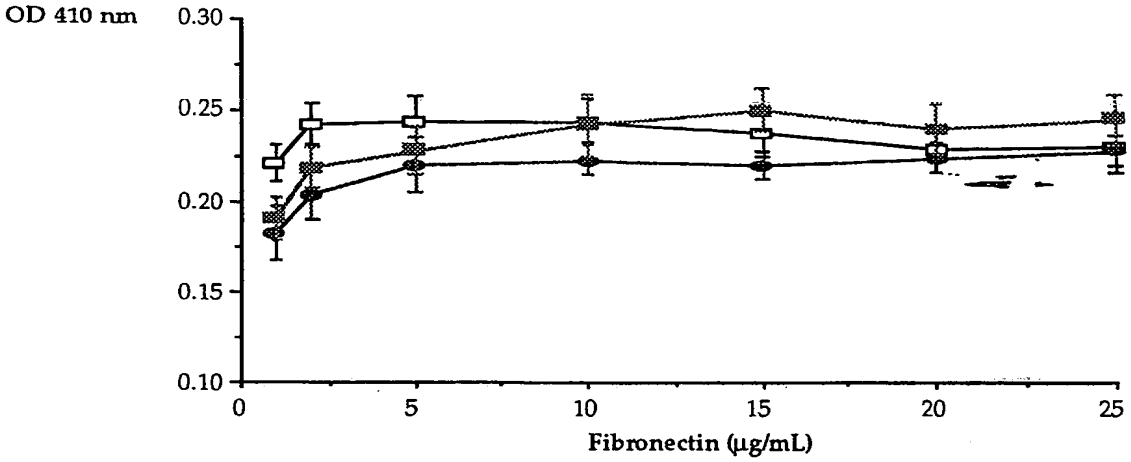
As previously stated, carbomer hydrogel preparations containing fibronectin concentrations higher than 0.2% fibronectin were prepared. As the method for the preparation of carbomer hydrogels containing concentrations of fibronectin higher than 0.2% included some modifications, we verified that the structural and functional activity of fibronectin was not affected by the new procedures used (e.g. lyophilization of fibronectin, dissolution in deionized water at 37°C for periods varying from 30 minutes to 1 hour, longer and more vigorous agitation). These assays were performed after a few days of storage at 4°C. Assays for stability following longer periods of storage have been performed after 12 months of storage at 4°C.

Results of SDS-PAGE revealed complete structural integrity of fibronectin extracted from 0.5 to 1% fibronectin carbomer hydrogels (Figure 15). This fibronectin also revealed equal gelatin-binding, cell adhesion and chemotactic promoting activity to freshly purified plasma fibronectin (Figures 16 and 17). For the cell adhesion bioassay, human virus-transformed fibroblast culture from skin (GM00637E, Coriel Institute for Medical Research, NJ) were used instead of newborn hamster kidney cells (BHK-21). However, since a new method (an immunofluorescence assay) for measuring opsonin activity of fibronectin was established, results using this method are described in the next section.



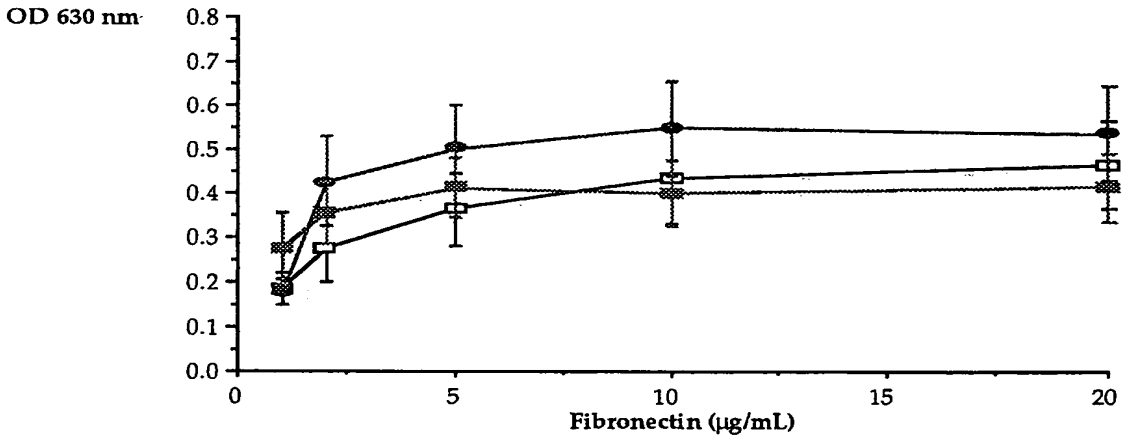
SDS-PAGE of different fibronectin preparations. Lane **A**: molecular weight standards 200 to 29 kDa. Lane **B**: fresh fibronectin preparation. Lane **C**: fibronectin extracted from a 0.5% fibronectin hydrogel specimen stored 12 months at 4°C. Lane **D**: : fibronectin extracted from a 1.0% fibronectin hydrogel specimen stored 12 months at 4°C. Protein bands were visualized with Coomassie brillant blue R250.

Figure 15: Structural stability of fibronectin in the carbomer hydrogels



Polystyrene microtiter plate was coated with gelatin and incubated with different dilutions of: (□) fresh preparation of fibronectin; (●) fibronectin extracted from 0.5% fibronectin carbomer hydrogel; (■) fibronectin extracted form 1.0% fibronectin carbomer hydrogel. Rabbit polyclonal antibody to human fibronectin and peroxidase labelled goat antibody to rabbit IgG and IgM were then respectively added to the wells. n = 6.

Figure 16: Gelatin-binding activity of fibronectin extracted from carbomer hydrogels stored at 4°C during 12 months



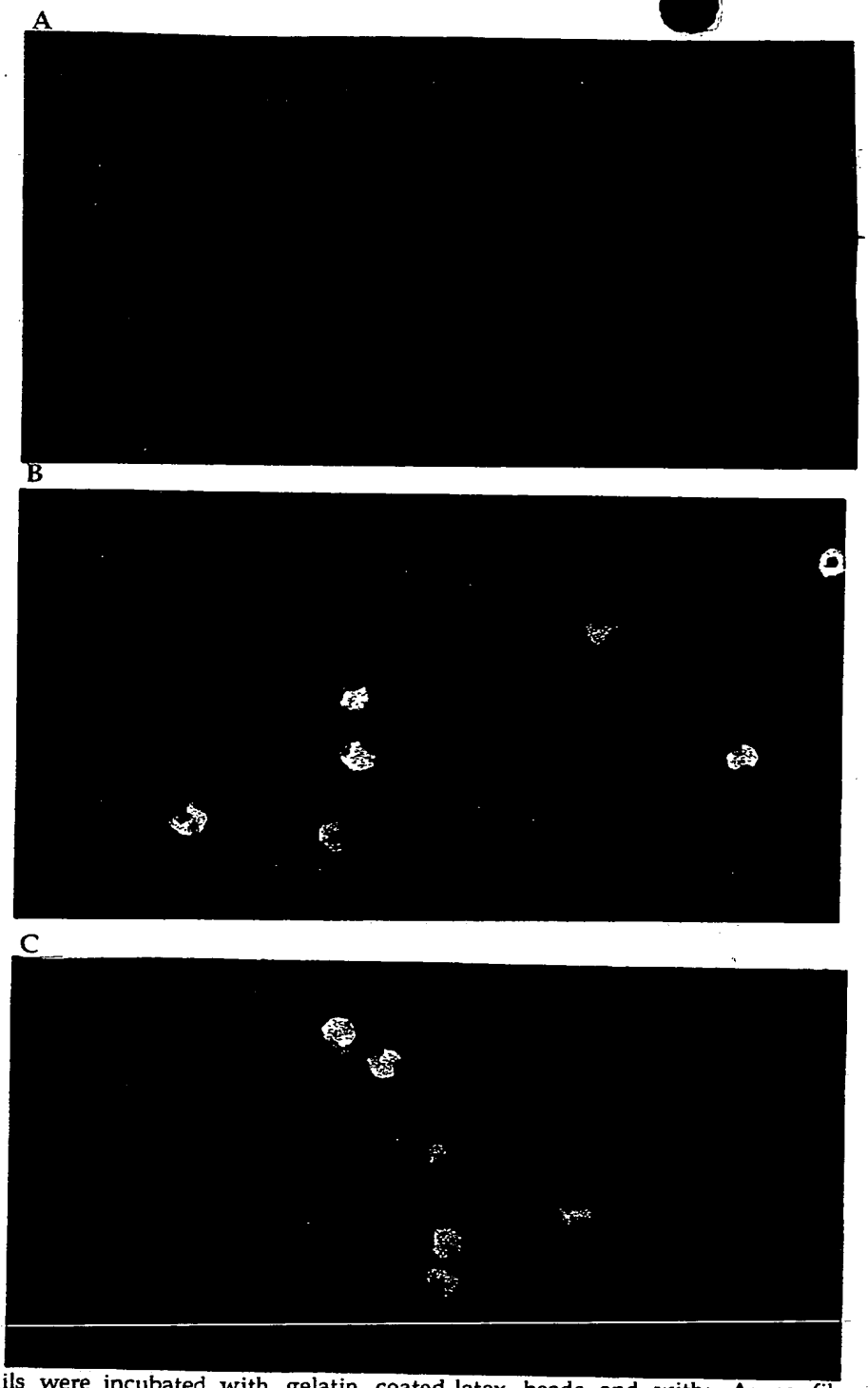
Polystyrene microtiter plate coated with gelatin is incubated with different dilutions of: (□) fresh preparation of fibronectin; (●) fibronectin extracted from 0.5% fibronectin carbomer hydrogel; (■) fibronectin extracted form 1.0% fibronectin carbomer hydrogel. GM00637E cells are next added to the wells for a period of 1 hour at 37°C. The cells are then fixed with paraformaldehyde and stained with toluidine blue. Attached cells were measured by absorbance at 630 nm. n=6.

Figure 17: Cell adhesion promoting activity of fibronectin from carbomer hydrogels stored at 4°C during 12 months

3.3.1 Immunofluorescence assay for measuring opsonin activity of fibronectin in a 1% fibronectin carbomer hydrogel

An immunofluorescence procedure was used to assess the capacity of fibronectin to stimulate the phagocytosis of gelatin-coated latex beads by human neutrophils. This assay was performed as described above except that gelatin was not radiolabelled. Furthermore, after centrifugation, human neutrophils were resuspended in 100 µL of PBS pH 7.5 and 10 µL of this suspension were layered on a cover slip and dried under air. Human neutrophils were then fixed with 0.5% formaldehyde in PBS for 30 minutes at room temperature. Cells were then incubated with mouse monoclonal antibody to human fibronectin (Sigma , MI) diluted 1/200, followed by an incubation with a FITC labelled goat antibody to mouse Ig (Sigma, MI) diluted 1/50. Both reactions were conducted for 30 minutes at room temperature. After three PBS washings, the coverslips were mounted in an aqueous mounting medium containing phosphate buffered glycerol and viewed under a Leitz Photo microscope with epifluorescence optics.

As shown in Figure 18 and 19, no difference was observed between freshly purified fibronectin and fibronectin extracted from a carbomer hydrogel into which 1 % fibronectin had been incorporated and stored 12 months at 4°C. This demonstrates that the processes by which higher concentrations of fibronectin are incorporated into the hydrogel do not affect fibronectin structural and functional stability.

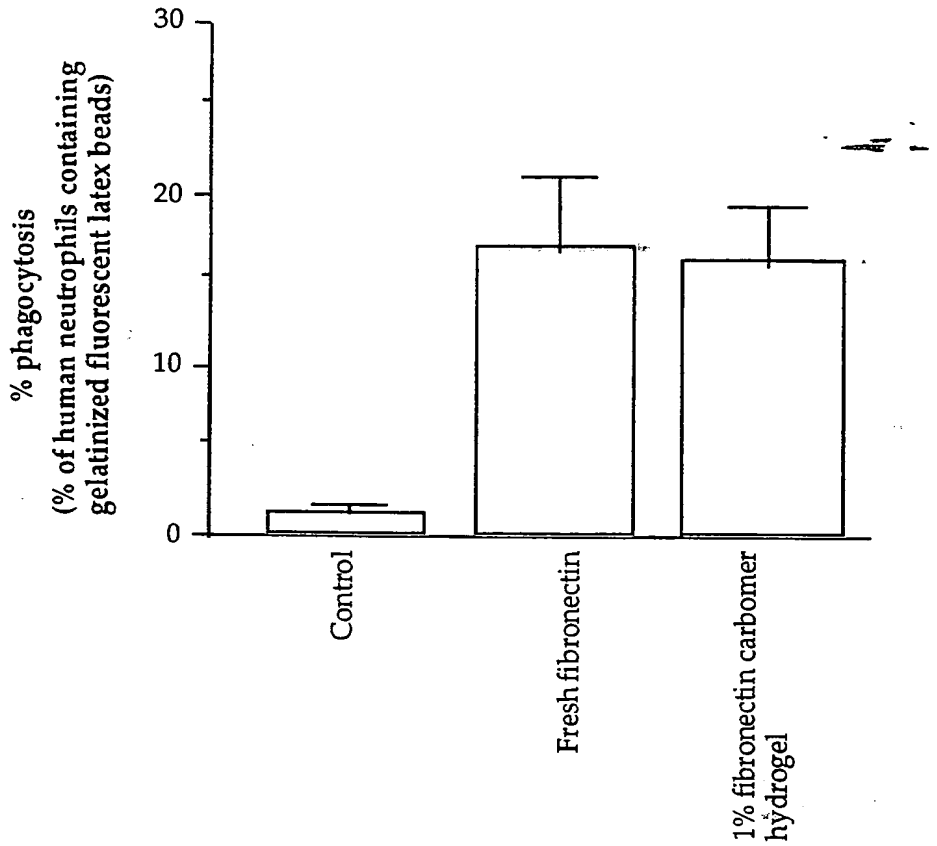


Human neutrophils were incubated with gelatin coated-latex beads and with: A: no fibronectin (negative control); B: fresh preparation of fibronectin; C: fibronectin extracted from a 0.5% fibronectin carbomer hydrogel stored 12 months at 4°C. Human neutrophils were layered on a cover slip and fixed with formaldehyde. Mouse monoclonal antibody to human fibronectin and FITC labelled goat antibody to mouse Ig were respectively added. Magnification 400 X.

Figure 18: Immunofluorescence assay for measuring opsonin activity of fibronectin

3.3.2 Flow cytometry assay for measuring opsonin activity of fibronectiin in a 1% fibronectin carbomer hydrogel

In order to obtain quantitative data, a flow cytometric procedure was used to assess the capacity of fibronectin to stimulate the phagocytosis of gelatin-coated fluorescent latex beads (Sigma, MI) by human neutrophils. Coupling of gelatin to carboxylated fluorescent latex beads with a mean diameter of 1.08 µm was performed according to Molnar et al. (1987) and as previously described. For the test, fluorescent gelatin-latex beads (125 µL from a 10% v/v in PBS pH 7.4) were added to a mixture containing 100 µL of heparin (100 U/mL), 300 µL of plasma fibronectin-depleted human serum to which were added either 100 µg of fresh preparation of fibronectin in PBS pH 7.4 or extracted from the 0.5% fibronectin carbomer hydrogel stored at 4°C during 12 months. Normal human plasma was deplateleted by centrifugation at 2000 RPM during 20 minutes and depleted of plasma fibronectin by chromatography on gelatin-Sepharose (1 ml of serum/mL gel bed). The mixture was preincubated at 37°C, usually two minutes, in a 12 X 75 mm polystyrene tube. Human neutrophils (300 µL of a suspension containing 2 X 10^7 cells/mL) were added and the incubation was continued for one hour at 37°C. Beads phagocytized by neutrophils are separated from free gelatin-coated fluorescent latex beads by two rapid centrifugations at 800g for 1 minute. Human neutrophils were then fixed with 1.0% formaldehyde in PBS. The fluorescence associated with neutrophils was evaluated with a flow cytometer (EPICS-XL, Coulter, FL, USA). As shown in Figure 19, no difference was observed between freshly purified fibronectin and fibronectin extracted from a 0.5% carbomer hydrogel stored at 4°C during 12 months (P = 0.3937).

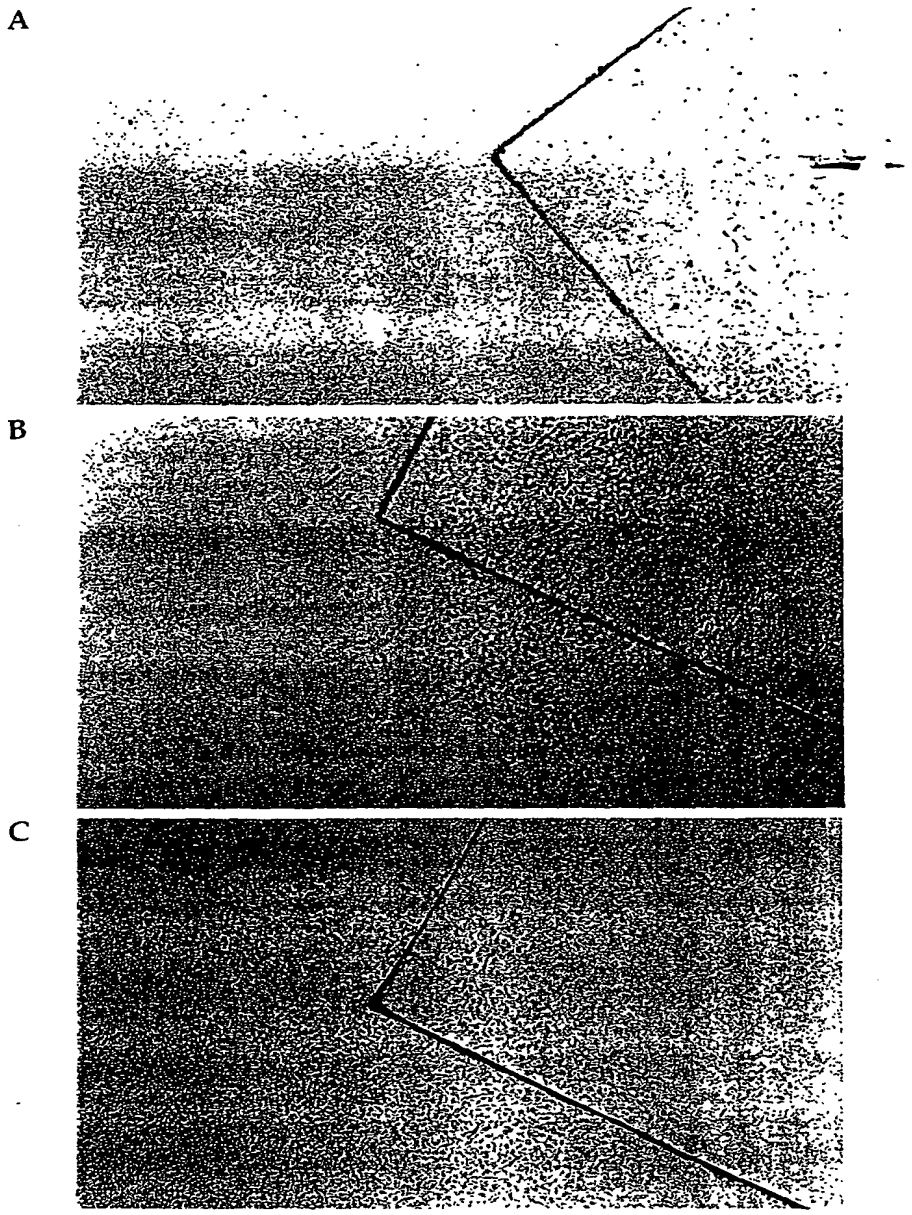


Human neutrophils were incubated with gelatin-coated fluorescent latex beads and with fresh plasma fibronectin-depleted with 100 µg of fresh fibronectin or 100 µg of fibronectin extracted from 1.0% fibronectin carbomer hydrogel. Beads phagocytized by neutrophils are separated from free gelatin-coated fluorescent latex beads by two rapid centrifugations. The fluorescence associated with neutrophils was evaluated with a fluorescent microscopy. Negative control contains no fibronectin and no fibronectin-depleted plasma. n=3; $P = 0.3937$ (Student's two-tailed t tests). Bars represent standard errors of the mean.

Figure 19: Opsonin activity of fibronectin extracted from a 1.0 % fibronectin carbomer hydrogel stored at 4°C during 12 months

3.3.3 Cell migration promoting activity of fibronectin

The promotion of cell migration is another intrinsic property of fibronectin. According to Hynes (1990), cell migration is either promoted when fibronectin is added directly to the cell cultures, or when surfaces are precoated with fibronectin. The present assay was performed using plastic petri dishes of 57 cm^2 surface area, coated with 6 mL of different preparations of fibronectin at 50 µg/mL in 50 mM sodium carbonate buffer pH 9.6 for 16 hours at 4°C. Human virus-transformed fibroblast cells (GM00637E) were cultured in six well polystyrene plates with 3 mL of DMEM medium containing 10% fetal calf serum at 37°C in 5% CO_2. Prior to the incubation of cells, a 1.8 mm^2 cover glass was placed in the bottom of each well. After 48 hours, a confluent culture of fibroblast cells was obtained on the cover glass. The cover glass was then washed with PBS pH 7.4 to remove the non-attached cells and placed in the bottom of fibronectin-coated petri dishes. Nine mL of DMEM medium with 10% of fetal calf serum, were added to petri dishes and incubated at 37°C in 5% CO_2. Cell migration was normally observed after 5 to 6 days. As shown in Figure 20, cell migration promoting activity is observed with freshly purified fibronectin (Figure 20 B), and fibronectin extracted from a 1 % fibronectin carbomer hydrogel stored at 4°C during 12 months (Figure 20 C). As shown in Figure 20 A, the carbomer hydrogel alone did not promote cell migration.



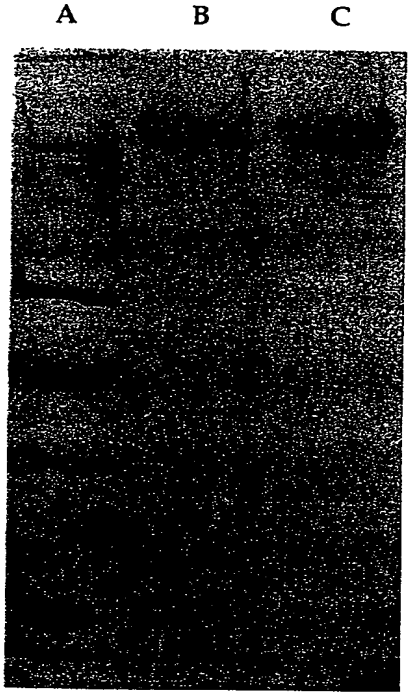
Plastic petri dishes were coated with: **A**: carbomer hydrogel alone; **B**: fresh preparation of fibronectin; **C**: fibronectin extracted from a 1.0% fibronectin carbomer hydrogel stored at 4°C during 12 months. A confluent culture of GM00637E cells adhered to a 1.8 mm^2 cover glass, was deposited in the bottom of petri dishes, and incubated in DMEM medium with 10% of serum at 37°C in 5 % CO_2 until migration was observed (between 5 to 6 days).

Figure 20: Chemotactic promoting activity of fibronectin extracted from a 1.0 % fibronectin carbomer hydrogel stored at 4°C during 12 months

3.4 Structural and functional stability of fibronectin extracted from calcium-alginate solid dressing containing 65 % (w/w) of fibronectin

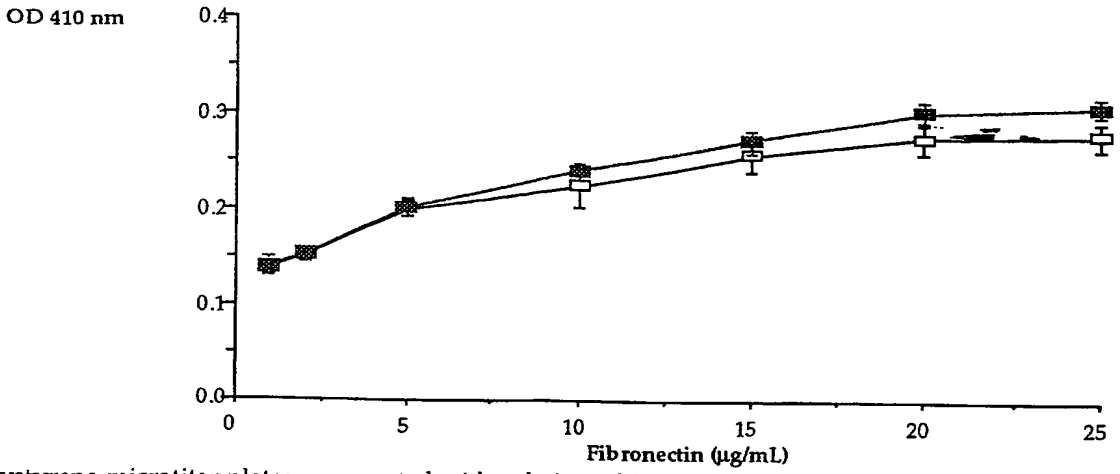
A calcium-alginate solid dressing containing fibronectin with concentration of 65 % (w/w) was prepared. The structural and functional activity of fibronectin were tested in order to verify that the procedures used (e.g. dissolution in deionized water at 37°C and at basic pH, adjustment at pH 4.0 with acetic acid, lyophilisation of fibronectin) did not affect fibronectin.

Results of SDS-PAGE revealed complete structural integrity of fibronectin extracted from a fibronectin-calcium alginate dressing (Figure 21). This fibronectin also revealed equal gelatin-binding, cell adhesion and chemotactic promoting activity to freshly purified plasma fibronectin (Figures 22, 23 and 24). All procedure have been previously described in sections 3.1 and 3.2



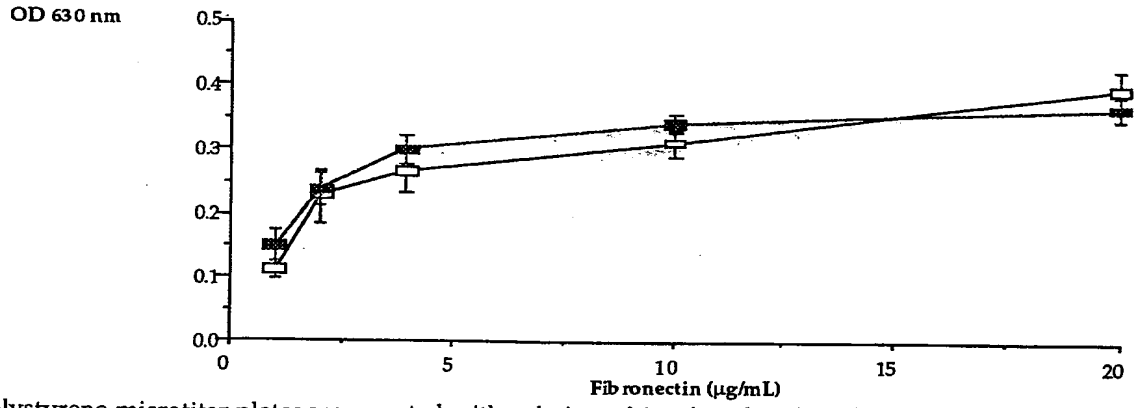
SDS-PAGE of different fibronectin preparations: Lane A: molecular weight standards 200 to 29 kD. Lane B: fresh preparation of fibronectin. Land C: fibronectin extracted from a calcium-alginate solid dressing. Proteins bands were visualized using the color-based silver stain method.

Figure 21: Structural stability of fibronectin in calcium-alginate solid dressing



Polystyrene microtiter plates were coated with gelatin and incubated with different concentrations of: (□) fresh preparation of fibronectin; (■) fibronectin extracted from the fibronectin-calcium alginate dressing. Rabbit polyclonal antibody to human fibronectin and peroxidase labelled goat antibody to rabbit IgG and IgM were then respectively added to the wells. n = 2. Bars represent standard errors of the mean.

Figure 22: Gelatin-binding activity of fibronectin extracted from the fibronectin-calcium alginate dressing

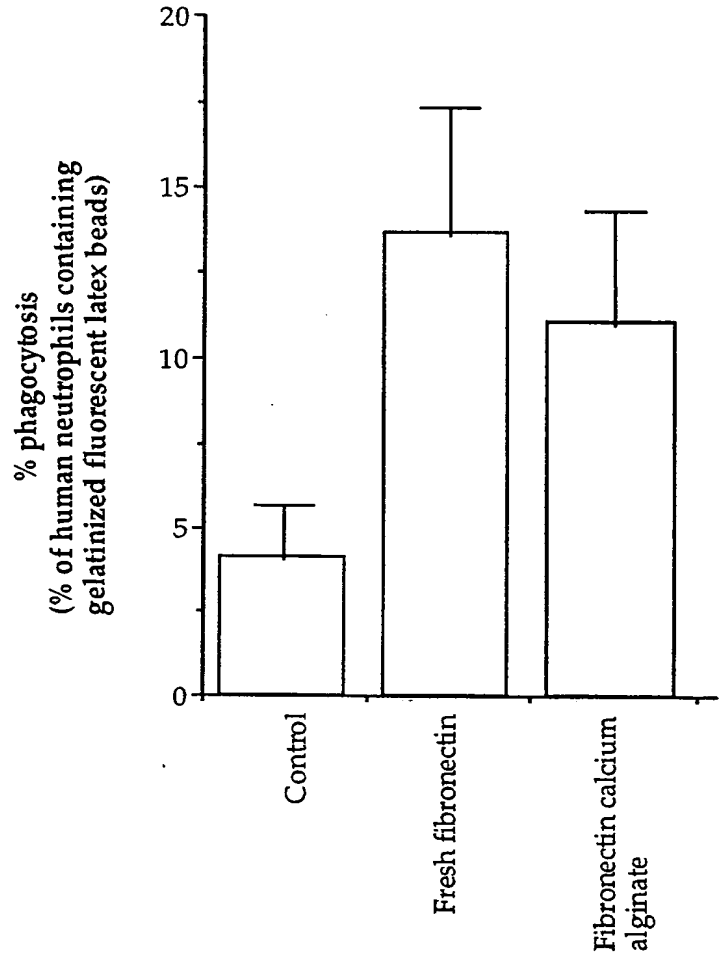


Polystyrene microtiter plates were coated with gelatin and incubated with different concentrations of: (□) fresh preparation of fibronectin; (■) fibronectin extracted from a fibronectin-calcium alginate dressing. GM00637E cells were next added to the wells for a period of 1 hour at 37°C. The cells were next fixed with paraformaldehyde and stained with toluidine blue. Attached cells were measured by absorbance at 630 nm. n=3. Bars represent standard errors of the mean.

Figure 23: Cell adhesion promoting activity of fibronectin extracted from the fibronectin-calcium alginate dressing

Plastic petri dishes were coated with: **A**: alginate alone; **B**: fresh preparation of fibronectin; **C**: fibronectin extracted from a fibronectin-calcium alginate dressing. A confluent culture of GM00637E cells adhered to a 1.8 mm^2 cover glass, was deposited in the bottom of petri dishes, and incubated in DMEM medium with 10% of serum at 37°C in 5 % CO_2 until migration was observed (between 5 to 6 days).

Figure 24: Chemotactic activity of fibronectin extracted from a fibronectin-calcium alginate dressing



Human neutrophils were incubated with gelatin-coated fluorescent latex beads and fibronectin-depleted fresh plasma. 100 µg of either freshly prepared plasma fibronectin or 100 µg of fibronectin extracted from fibronectin-calcium alginate dressing were next added to the mixture. Fluorescence was evaluated with by fluorescence microscopy. n=3; $P = 0.2970$ (Student's two-tailed t tests). Bars represent standard errors of the mean.

Figure 25: Opsonin activity of fibronectin extracted from the fibronectin-calcium alginate dressing

3.5 Dry heat treatment of plasma fibronectin (pasteurization)

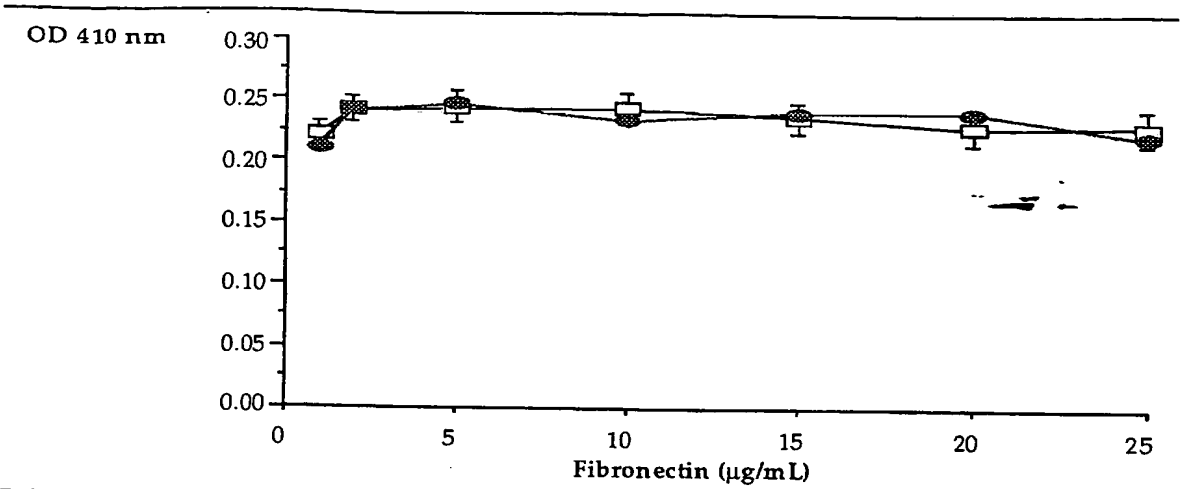
A viral inactivation solvent/detergent method (25°C for 6 h) is already performed on human homologous plasma (see section 1.2) and has been recognized to be highly effective in the destruction of enveloped-viruses. According to Radosevich (Seminars in thrombosis and hemostasis. 24 (2) 157-161, 1998), pasteurization has the advantage to having a broader virucidal action owing to its additional ability to inactivate non-enveloped viruses, usually more resistant to physicochemical treatments than enveloped viruses. Thus, improvements in viral safety for plasma products may be obtained by combining different viral reduction procedures (e.g. solvent-detergent + dry heat treatments). In order to meet the new recommendations in the plasma fractionation industry, we applied a dry heat treatment on lyophilized solvent-detergent treated-fibronectin at 68°C for 96 hours.

3.5.1 Structural and funtional stability of pasteurized fibronectin

When a viral inactivation/removal procedure is implemented, it is important to measure that there is no alteration of protein structure and biological activities. Results of SDS-PAGE revealed complete structural integrity of pasteurized fibronectin (Figure 26). This fibronectin also revealed equal gelatin-binding, cell adhesion promoting activity and chemotactic activity of freshly purified plasma fibronectin (Figures 27, 28 and 29).

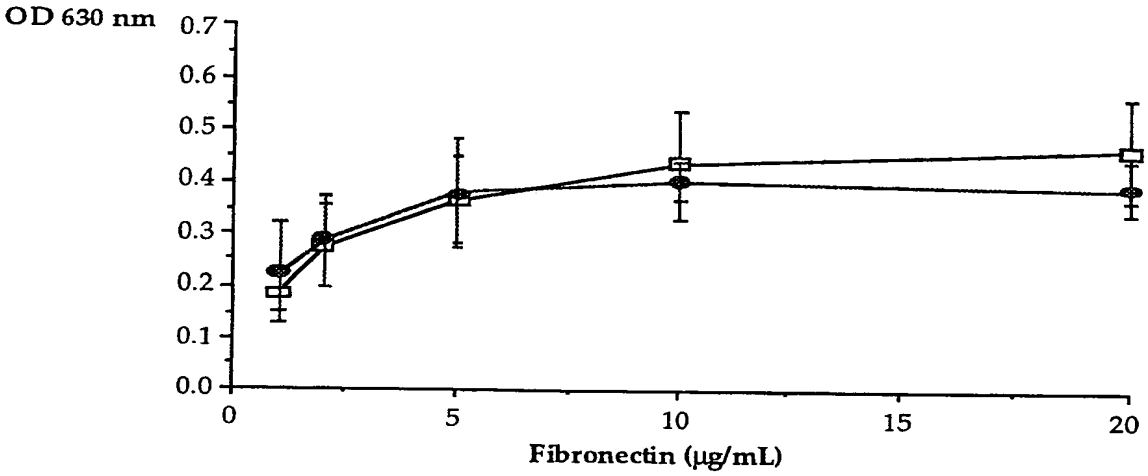
SDS-PAGE of different fibronectin preparations: Lane A: molecular weight standards 200 to 29 kD. Lane B: fresh preparations of fibronectin. Land C: pasteurized fibronectin at 68°C during 96 hours. Protein bands were visualized using the color-based silver stain method.

Figure 26: Structural stability of pasteurized human plasma fibronectin



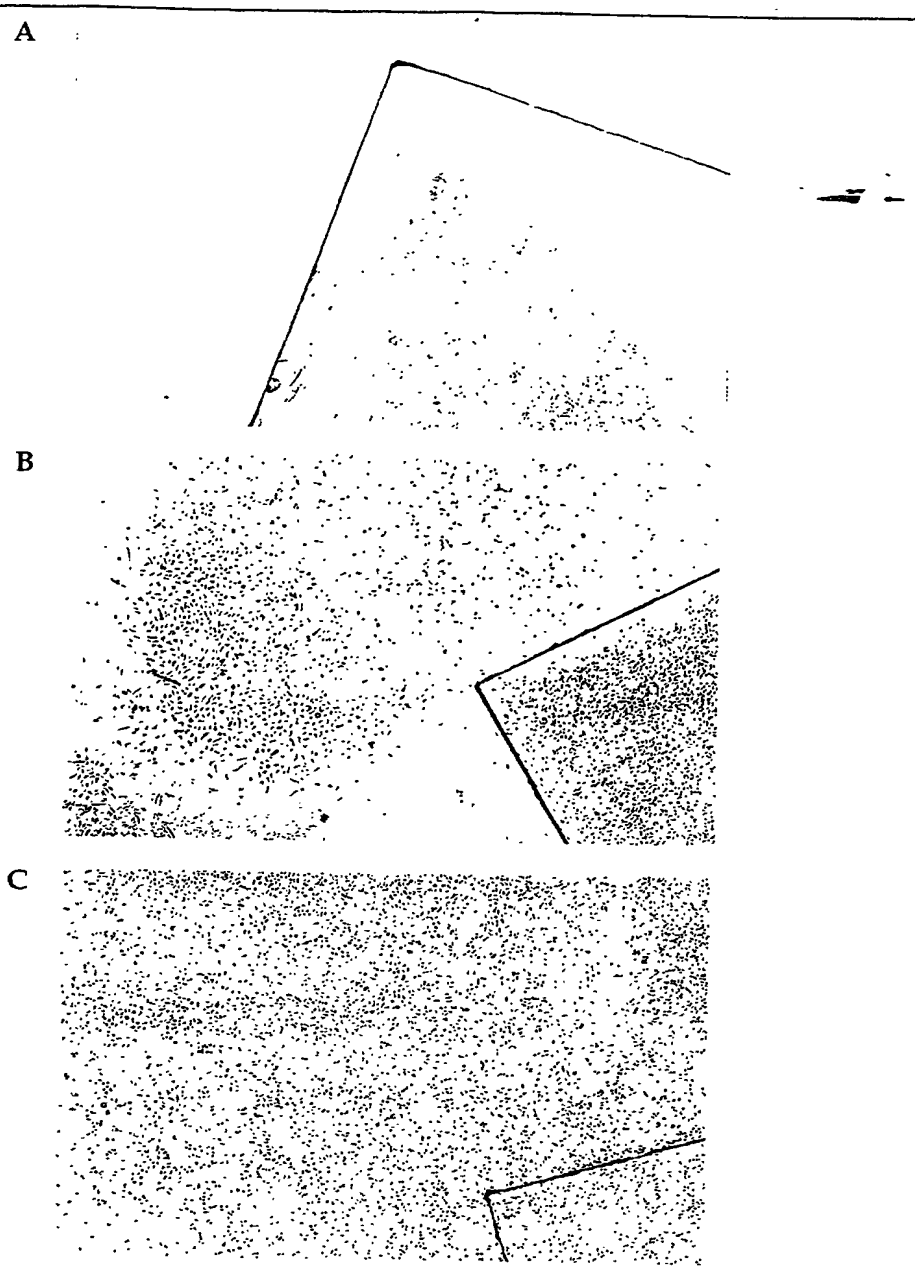
Polystyrene microtiter plate was coated with gelatin and incubated with different dilutions of: (□) fresh preparation of fibronectin; (●) pasteurized fibronectin with a dry heat treatment of 68°C/96 h. Rabbit polyclonal antibody to human fibronectin and peroxidase labelled goat antibody to rabbit IgG and IgM were then respectively added to the wells. n = 5. Bars represent standard errors of the mean.

Figure 27 : Gelatin-binding activity of pasteurized fibronectin compared to a fresh preparation of fibronectin



Polystyrene microtiter plate coated with gelatin is incubated with different dilutions of: (□) fresh preparation of fibronectin; (●) pasteurized fibronectin with a dry heat treatment of 68°C/96 h. GM00637E cells are next added to the wells for a period of 1 hour at 37°C. The cells are then fixed with paraformaldehyde and stained with toluidine blue. Attached cells were measured by absorbance at 630 nm. n=4. Bars represent standard errors of the mean.

Figure 28: Cell adhesion promoting activity of pasteurized fibronectin compared to a fresh preparation of fibronectin



Plastic petri dishes were coated with: A: no fibronectin (negative control); B: fresh preparation of fibronectin; C: pasteurized fibronectin with a dry-heat treatment at 68°C during 96 hours. A confluent culture of GM00637E cells adhered to a 1.8 mm^2 cover glass, was deposited in the bottom of petri dishes, and incubated in DMEM medium with 10% of serum at 37°C in 5 % CO_2 until migration was observed (between 5 to 6 days).

Figure 29 : Chemotactic activity of pasteurized fibronectin

4. FIBRONECTIN AND WOUND HEALING

The study of the efficacy of the fibronectin-calcium alginate wound dressing in stimulating wound healing was performed using the rabbit ear dermal ulcer model of wound healing as developed by Mustoe et al. (1991) J. Clin Invest 87: 694-703.

4.1 The rabbit ear dermal ulcer model

Young adult New Zealand white rabbits, 3.0-3.5 kg (Charles River Laboratories, Canada) were anesthetized with ketamine (60 mg/kg) and xylazine (95 MG/kg). Using a 6-mm trephine and microsurgical instruments, four circular full-thickness 6-mm dimeter ulcers were made to the depth of bare cartilage under sterile conditions. The solid dressings were applied once at the time of surgery as a piece of 6 mm in diameter. Alginate dressing (Kaltostat, ConvaTec, Skillman, NJ), collagen-alginate dressing (Fibracol, Johnson & Johnson, Arlington, TX) and fibronectin-calcium alginate dressing were applied on the ulcers and wetted with 40 µL of saline solution. Control treatment (identified in Figures 31, 32, 33, 34) were ulcers treated with 40 µL of saline alone. The wounds were covered with an occlusive polyurethane film (Tegaderm; 3M, Minneapolis, MN) to prevent wound dessication. Neck collars were placed on rabbits for the duration of the experiment. Differences in rates of healing between treatment groups were measured at days 7. At the time of killing, the ulcers were bisected and fixed in 10% buffered formalin. The specimens were then dehydrated in graded alcohol and xylene, embedded in paraffin, and sectioned, taking care to obtain a cross section as near as possible to the center of the wound. After Masson-trichrome staining of 3-µm sections, the granulation tissue gap (GTG) and the maximum height (MH) of granulation tissue at the advancing edges of the wound were measured by histomorphometry using Biometrics Bioquant true color laser vision (R&M, Nashville, TN).

4.2 Histomorphometric measurement of new granulation tissue

Calculations of the mean maximum height (MH) of the new granulation tissue and the new granulation volume (NGV) were made as described in Figure 30. The GAP

distance is used to measure area of wound $(GAP/2)^2\pi$. On day 0 (day of surgery), the measured GAP was 6.2 mm and the area was $(6.2/2)^2\pi = 30.19$ mm^2. New granulation tissue area is area of wound at day 0 minus area of wound at day 7-8. The new granulation volume is new tissue area x MH. Area and volume measurements for new granulation tissue were calculated based on the assumptions that the wounds healed concentrically and did not contract. Statistical analysis was carried out using a Student's paired *t* test for each formulations studied using Excel version 5.0 (Microsoft Corporation). All comparisons were made to paired control wounds (saline, alginate or collagen-alginate). $P < 0.05$ was considered significant.

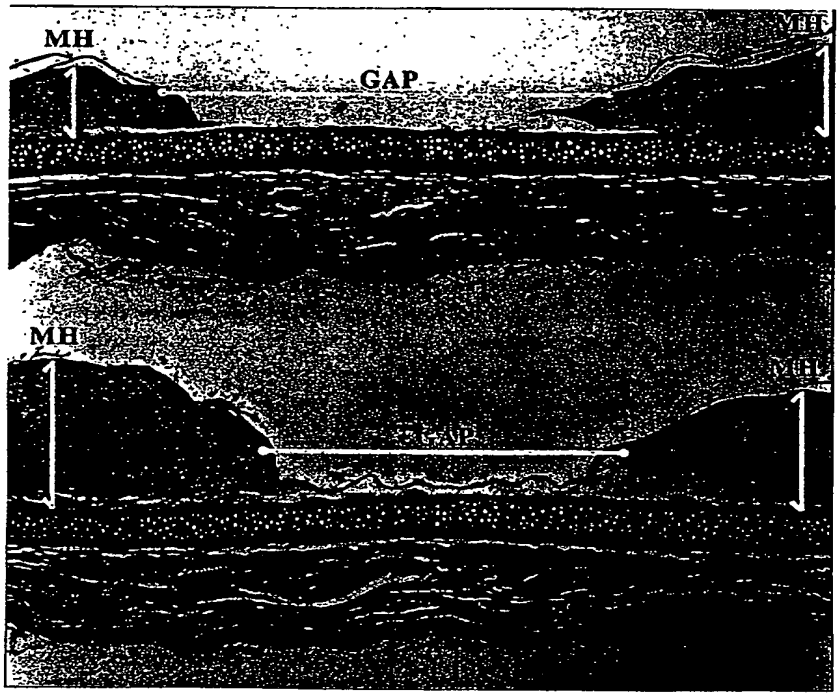
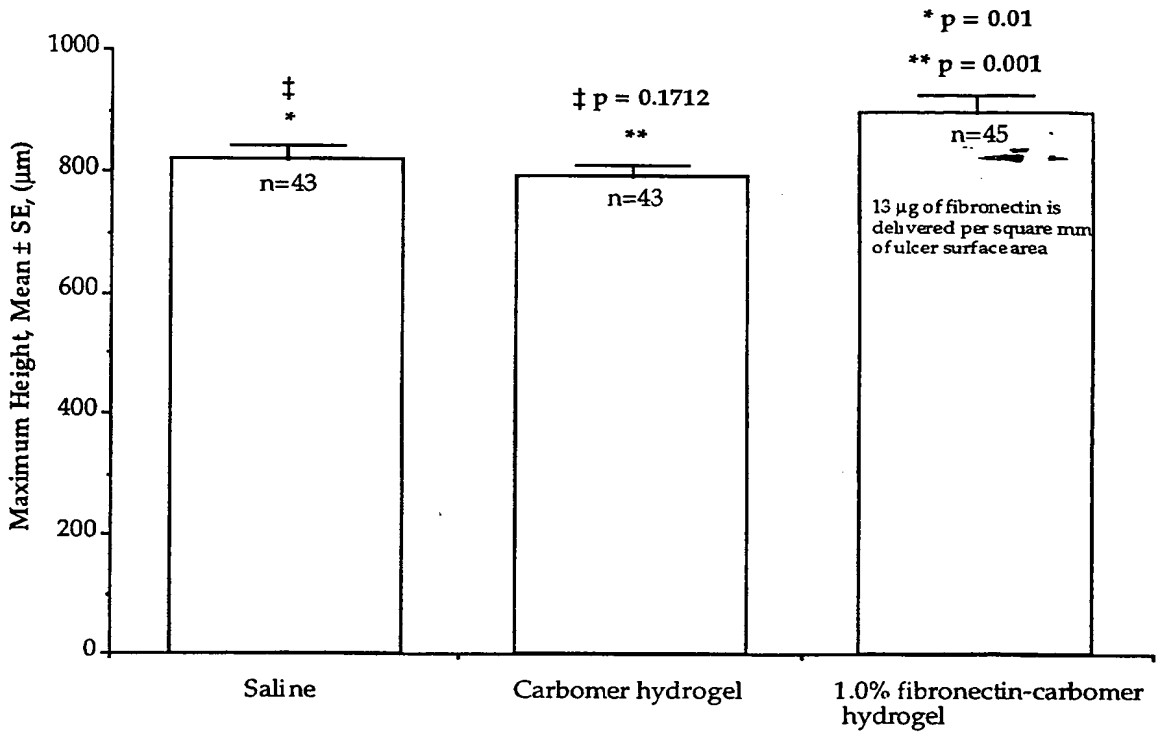


Figure 30: Histomorphometric measurement of new granulation tissue

4.3 Effect of 1.0% fibronectin-carbomer hydrogel

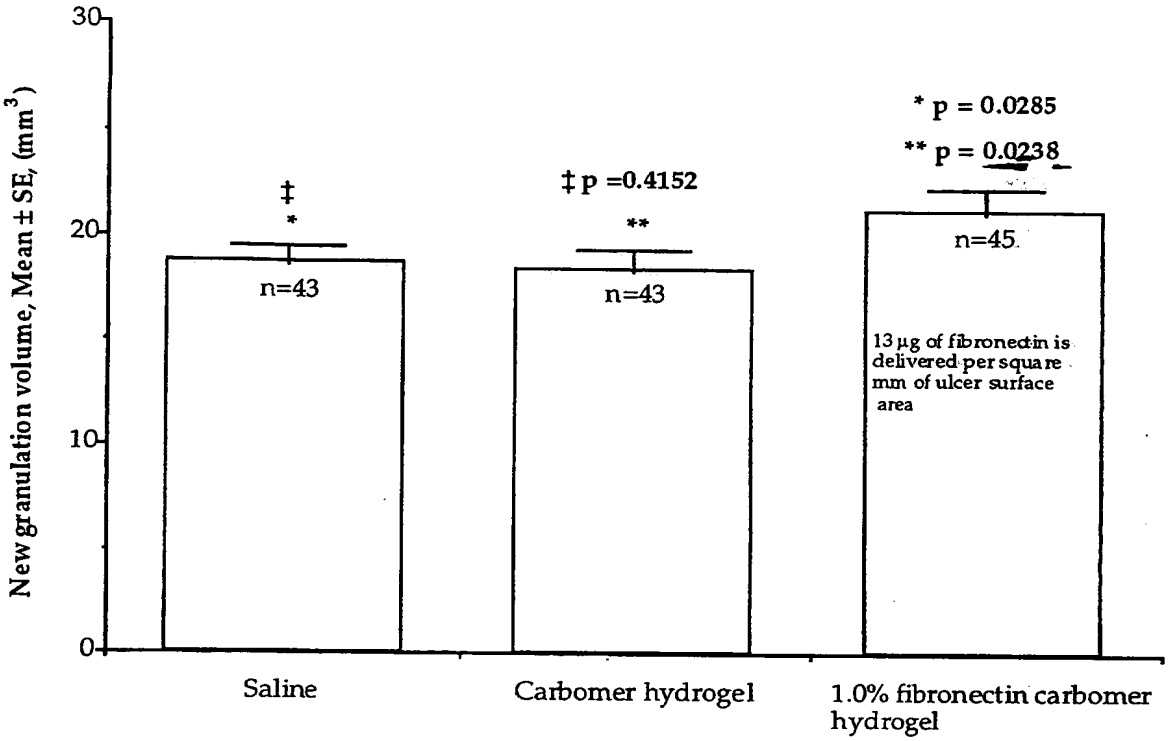
Treatment with fibronectin in a 1.0% fibronectin-carbomer hydrogel formulation on the rabbit dermal ulcer model was evaluated in its capacity to stimulate the formation of new granulation tissue. The fibronectin was applied as a 0.281%

carbomer hydrogel, applying a volume of 40µL of hydrogel containing 400 µg of fibronectin. This delivered approximately 13 µg of fibronectin per mm^2 rabbit ulcer surface area. A once a day application was made for a period of 7 days. The volume of topical formulations appplied to the control wounds (saline or 0.281% carbomer hydrogel containing no fibronectin) was also 40 µL. Antiseptic tulle gras dressing (Bactigras®, Smith & Nephew, Lachine ., Canada) and a non-adherent absorbent dressing (Melolite®, Smith & Nephew, Lachine , Canada) were used to prevent wound dessication. Before each daily application, the ulcers were washed with sterile saline solution, gently cleaned with a moistened cotton-swab. Histomorphometric measurements revealed that the mean maximum height of new granulation tissue in fibronectin-carbomer hydrogel-treated wounds (900 ± 30 µm, n=45) was significantly higher when compared to treatment with either saline (819 ± 23 µm, n=43, P = 0.01) or carbomer hydrogel containing no fibronectin (793 ± 19 µm, n=43, P = 0.001), (Figure 31). Furthermore, the volume of new granulation tissue formation was significantly greater with 1.0% fibronectin-carbomer hydrogel treatment (21.22 ± 1.14 mm^3, n=45) when compared to saline (18.67 ± 0.79 mm^3, n=43, P = 0.0285) or carbomer hydrogel containing no fibronectin (18.43 ± 0.95 mm^3, n=43, P = 0.0238, Figure 32). No difference was observed between saline and carbomer hydrogel alone (P = 0.4152).



New granulation tissue formation measured as maximum height (µm) in response to 1.0% fibronectin carbomer hydrogel after 7 days of treatment. The fibronectin was applied as a 0.281% carbomer hydrogel (40µL containing 400 µg of fibronectin) at the time of the surgery and daily during 7 days. The volume of the topical formulations applied to control wounds (saline and 0.281% carbomer hydrogel) was also 40 µL. Antiseptic tulle gras dressing (Bactigras) and non-adherent absorbent dressing (Melolite) were used to prevent wound dessication. The 1.0% fibronectin-carbomer hydrogel treatment (n =45) was significantly better than either saline (P = 0.01, n=43) or carbomer hydrogel containing no fibronectin in stimulating new granulation tissue formation (P = 0.001, n=43). No difference was observed between saline and carbomer hydrogel alone (P = 0.1752). Student two-tailed t test. Bars represent standard errors of the mean.

Figure 31: New granulation tissue formation measured as maximum height (µm) in response to 1.0% fibronectin carbomer hydrogel after 7 days of treatment

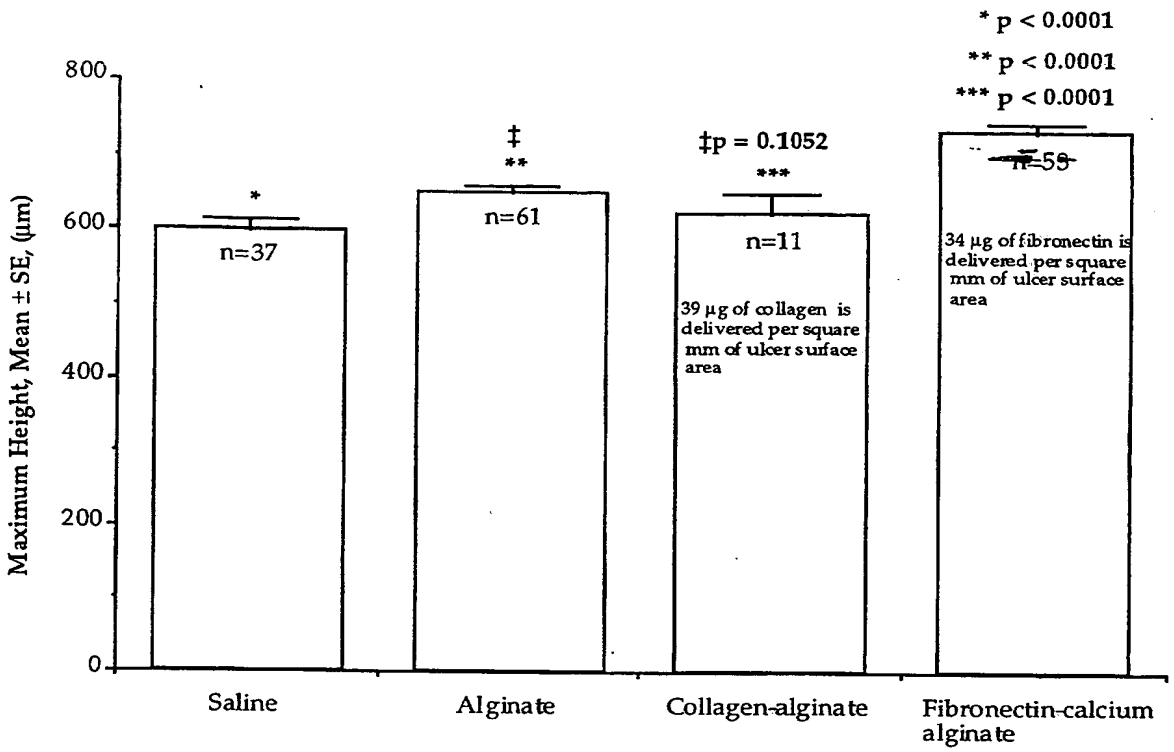


New granulation volume in response to 1.0% fibronectin carbomer hydrogel after 7 days of treatment. The fibronectin was applied as a 0.281% carbomer hydrogel (40µL containing 400 µg of fibronectin) at the time of the surgery and daily during 7 days. The volume of the topical formulation applied to the control wounds (saline and 0.281% carbomer hydrogel) was also 40 µL. Antiseptic tulle gras dressing (Bactigras) and non-adherent absorbent dressing (Melolite) were used to prevent wound dessication. The 1.0% fibronectin-carbomer hydrogel treatment (n=45) was significantly better when compared to saline ($P = 0.0285$, n=43) and carbomer hydrogel containing no fibronectin ($P = 0.0238$, n=43) in stimulating new granulation tissue formation. No difference was observed between saline and carbomer hydrogel alone ($P = 0.4152$). Student two-tailed t test. Bars represent standard errors of the mean.

Figure 32: New granulation volume in response to 1.0% fibronectin carbomer hydrogel after 7 days of treatment

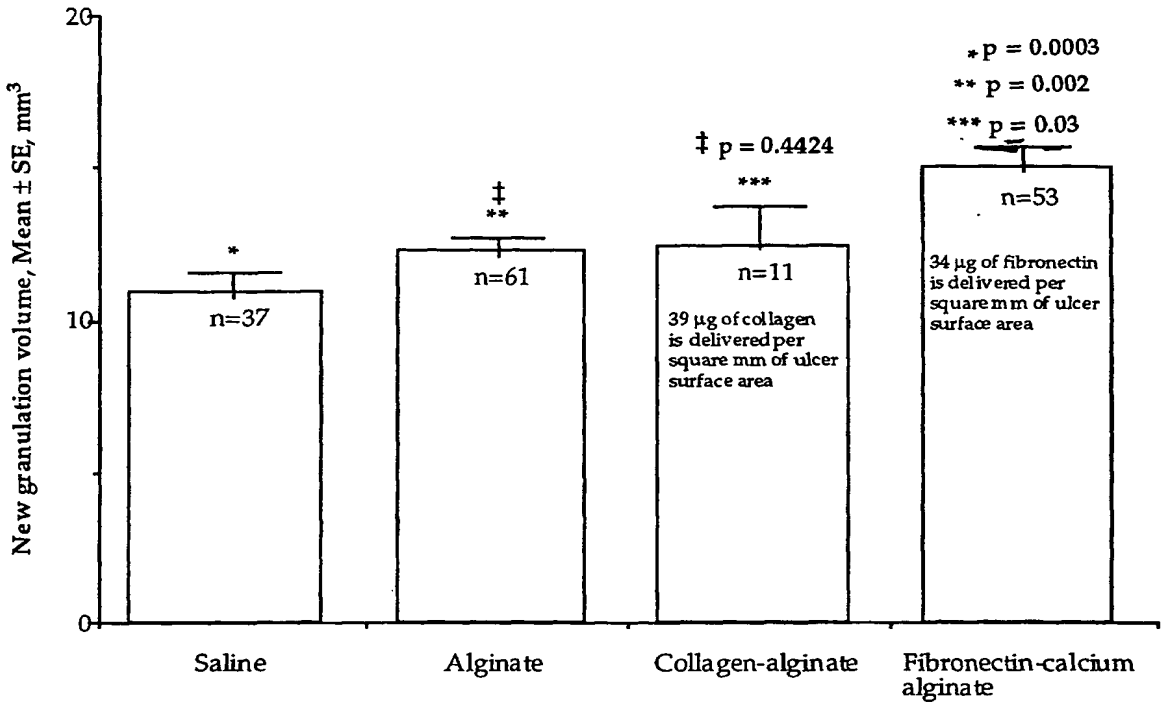
4.4 Effect of fibronectin-calcium alginate dressing containing 65 % fibronectin (w/w) in stimulating new granulation tissue formation

Dressings were applied once at the time of surgery as a piece of 6-mm in diameter. Either alginate (Kaltostat®, ConvaTec, Skillman, NJ), collagen-alginate (Fibracol®, Johnson & Johnson, Arlington, TX) or fibronectin-calcium alginate dressings were placed on the ulcers and wetted with 40 µL of saline solution. Control treatment (identified as "saline") were ulcers treated with 40 µL of saline alone. The wounds were covered with an occlusive polyurethane film (Tegaderm®; 3M, Minneapolis, MN) to prevent wound dessication. Results of histomorphometric measurements show that the effect of a single application of the fibronectin-calcium alginate dressing was significantly better than alginate (Kaltostat) or collagen-alginate (Fibracol) dressings in generating new granulation tissue in the rabbit ear dermal ulcer model. This amount of fibronectin delivered represents approximately 34 µg per mm^2 rabbit surface ulcer area whereas a dose of 39 µg per mm^2 of collagen was used for collagen-alginate dressing. As shown in Figure 33, after 7 days of treatment, the maximum height (MH) for fibronectin-calcium alginate treated wounds was significantly higher compared to the control wounds (732 ± 13 µm vs 599 ± 16 for saline, 648 ± 11 for alginate and 621 ± 27 for collagen-alginate treated wounds, $P < 0.0001$ in all cases). For the new granulation volume values (NGV), significant increases (more than 120% compared to collagen-alginate, 122% compared to alginate and 137% compared to saline) were observed (Figure 34). The calculated values were respectively 15.02 ± 0.77 mm^3 for fibronectin-calcium alginate, 10.95 ± 0.76 for saline, 12.29 ± 0.59 for alginate and 12.46 ± 1.47 collagen-alginate. Neither alginate nor collagen-alginate dressings were significantly different for the MH values ($P = 0.1052$) and NGV values ($P = 0.4424$) when compared to each other or to saline.



New granulation tissue formation measured as maximum height (µm) in response to treatment with a fibronectin-calcium alginate dressing after 7 days of treatment. Dressings were applied once at the time of surgery as a piece of 6 mm in diameter. Alginate dressing (Kaltostat), collagen-alginate dressing (Fibracol) and fibronectin-calcium alginate dressing were placed on the ulcers and wetted with 40 µL of saline solution. Control treatment (identified as "saline") were ulcers treated with 40 µL of saline alone. Occlusive dressings (Tegaderm) were used to prevent wound dessication. The fibronectin-calcium alginate dressing (n=53) was significantly better than saline ($P < 0.0001$, n=37), alginate ($P < 0.0001$, n=61) or collagen-alginate ($P < 0.0001$, n=11). No difference was observed between alginate and collagen-alginate dressing ($P = 0.1052$). Student two-tailed t test. Bars represent standard errors of the mean.

Figure 33: New granulation tissue formation measured as maximum height (µm) in response to treatment with a fibronectin-calcium alginate dressing after 7 days of treatment



New granulation volume in response to treatment with fibronectin-calcium alginate dressing after 7 days of treatment. Dressings were applied once at the time of surgery as a piece of 6 mm in diameter. Alginate (Kaltostat), collagen-alginate (Fibracol) and fibronectin-calcium alginate dressings were placed on the ulcers and wetted with 40 µL of saline solution. Control treatment (identified as "saline") were ulcers treated with 40 µL of saline alone. Occlusive dressings (Tegaderm) were used to prevent wound dessication. The fibronectin-calcium alginate dressing (n=53) was significantly better compared to saline ($P < 0.0003$, n=37), alginate ($P < 0.002$, n=61) or collagen-alginate ($P < 0.03$, n=11) dressing in stimulating new granulation tissue formation. No difference was observed between alginate and collagen-alginate dressing ($P = 0.4424$). Student two-tailed t test. Bars represent standard errors of the mean.

Figure 34: New granulation volume in response to treatment with a fibronectin-calcium alginate dressing after 7 days of treatment

5. CLINICAL STUDY-PROTOCOL FN-95-01: A RANDOMIZED, DOUBLE BLIND, PLACEBO CONTROLLED TRIAL ON EFFICACY AND SAFETY OF TOPICALLY APPLIED HOMOLOGOUS HUMAN PLASMA FIBRONECTIN IN THE TREATMENT OF CHRONIC VENOUS LEG ULCERS

A clinical study was conducted in 46 patients with at least one venous chronic leg ulcer. A major objective of this study was safety data acquisition comparing two treatment groups: 1-patients treated with carbomer hydrogel alone; 2- patients treated with carbomer hydrogel containing 0.2% fibronectin. The study was double-blinded, randomized and was performed in four different wound healing centers in accordance with Good Clinical Practice (GCP) guidelines. The treatment consisted of a twice a day application at the wound site of either carbomer hydrogel alone or carbomer hydrogel containing 0.2% fibronectin. Sufficient hydrogel was applied to produce a 0.5 mm coating of the wound surface area. Before each topical application, the wound was washed with sterile water. The protocol describing inclusion and exclusion criteria, as well as conduct of the study is presented in Annex B.

5.1 Extent of exposure

Twenty six patients were treated with the carbomer hydrogel containing 0.2% of fibronectin for a mean exposure time ot 11.5 ± 2.3 weeks. Adverse experiences were compared to 20 other patients who had received the carbomer hydrogel alone for a mean exposure time of 9 ± 2.6 weeks.

5.2 Serious adverse events

No serious adverse event was reported during study.

5.3 Other adverse events

5.3.1 Investigator and patient reported adverse events

One patient was hospitalized one month after study termination for skin wound infection and was treated with intravenous antibiotics. This patient was in the fibronectin hydrogel group and the topical batch preparation used to treat this particular patient was tested as sterile.

Three categories of symptoms were reported by patients: 1- burning sensation at wound site; 2- tingling sensation at wound site; 3- shooting pain at wound site. Complaints were graded as follows: Very light; Light; Moderate; Severe; Very Severe.

When comparing the percentages of patients reporting symptoms in any of the above categories, no significant difference was observed between both treatment groups (Table 2). Furthermore, the percentages of patients reporting these symptoms at screening visit, baseline and during study was not significantly different.

Table 2: Individual patient reporting of adverse events

A. Fibronectin carbomer hydrogel group

Patient #	Wound symptoms n=26								
	Screening visit			Baseline visit*			End of treatment		
	B	T	SP	B	T	SP	B	T	SP
GF-101	VL	-	-	S	-	-	VS	-	-
LM-110	S	-	-	S	-	S	VS	-	-
SD-111	-	-	-	-	-	-	-	-	-
HT-113	-	-	-	-	-	-	-	-	-
LF-115	M	-	-	VS	-	-	VS	-	-
JC-116	-	-	S	-	-	-	-	-	-
MLL-118	-	-	-	-	VL	-	-	-	-
MG-120	-	-	-	-	-	-	-	-	-
YJ-123	-	-	-	-	-	-	-	-	-
FV-124	-	-	-	L	-	-	L	-	-
MD-127	L	-	-	-	-	-	-	-	-
EV-130	L	-	-	-	-	M	M	-	M
JGB-131	S	-	S	L	-	L	M	M	M
JK-132	M	M	M	L	L	-	L	L	-
CR-133	-	-	L	-	-	L	-	-	-
LB-140	L	L	-	L	-	-	S	S	-
AL-141	-	-	-	-	-	-	VL	-	-
RT-150	S	-	-	M	-	-	L	-	-
RL-153	VS	-	VS	L	-	-	-	-	-
TP-155	M	-	-	M	-	-	L	-	L
LP-156	VS	-	VS	M	M	-	-	-	-
LPS-157	M	-	M	S	-	M	L	-	L
LB-163	-	-	L	L	-	-	-	-	-
JK-166	L	-	-	L	-	-	-	-	-
LLA-167	VL	-	-	L	-	-	-	-	-
PD-180	L	-	-	L	-	-	-	-	-
(%)	62	8	31	62	12	19	46	12	15

B: Burning sensation
T: Tingling
SP: Shooting Pain
S: Severe
VS: Very Severe
VL: Very Light
L: Light
M: Moderate

*: All patients had received two weeks of treatment with carbomer hydrogel alone at this point (see protocol FN.95.01).

B. Carbomer hydrogel alone group

Patient #	Wound symptoms n=20								
	Screening visit			Baseline visit*			End of treatment		
	B	T	SP	B	T	SP	B	T	SP
LL-100	M	-	M	M	-	M	-	-	-
RV-114	-	-	S	-	-	M	-	-	-
BB-117	-	-	VL	VS	-	-	L	-	-
LL-121	-	-	L	M	-	-	VS	-	VS
GA-122	M	-	-	M	-	-	S	-	S
JH-125	-	-	-	VL	-	-	-	-	-
LD-126	L	-	-	-	-	-	-	-	-
RM-128	-	-	-	-	-	S	-	-	-
GT-129	M	-	-	L	-	-	-	-	-
CS-134	S	S	S	-	-	-	-	-	-
CAM-135	M	-	M	M	M	M	-	-	-
JC-151	M	M	M	L	-	-	VL	-	-
SC-152	M	-	M	M	-	-	L	-	L
MSA-154	-	-	-	-	-	-	-	-	-
GR-158	-	L	L	-	-	VL	-	-	-
CL-159	L	-	-	M	-	-	S	-	S
YC-160	L	-	L	L	-	L	L	-	L
SG-161	-	-	-	S	-	-	M	-	-
SM-164	-	-	-	-	-	-	-	-	-
RB-165	M	-	-	M	-	-	M	-	M
(%)	55	2	50	65	5	30	45	0	30

B: Burning sensation
T: Tingling
SP: Shooting Pain
S: Severe
VS: Very Severe
VL: Very Light
L: Light
M: Moderate

*: All patients had received two weeks of treatment with carbomer hydrogel alone at this point (see protocol FN.95.01).

5.3.2. Reasons for premature termination from study

Six patients were prematurely terminated from the study. Three were from the carbomer hydrogel alone group and three were from the fibronectin carbomer hydrogel group. Reasons for discontinuation are given in Table 3 for each patient.

Table 3: Reasons for premature termination from study

Patient #	Treatment	Reasons for premature termination	Terminated at week
121	CH	Very severe burning sensation and very severe shooting pain at wound site	2
122	CH	Severe burning sensation and severe shooting pain at wound site. Presence of necrosis around the ulcer was observed	4
165	CH	Moderate burning sensation and moderate shooting pain at wound site	8
123	FCH	Very poor compliance	8
130	FCH	Moderate burning sensation and moderate shooting pain at wound site	4
140	FCH	Severe burning sensation and severe tingling sensation at wound site	2

CH: Carbomer Hydrogel
FCH: 0.2% Fibronectin Carbomer Hydrogel

5.3.3. Plasma concentrations of fibronectin at the beginning and end of study

The purpose for measuring plasma concentrations of fibronectin in patients receiving treatment was to rule out the possibility that topical administration of fibronectin can affect levels of this protein in plasma. This is important in view of the fact that fibronectin can cause fibrosis of tissues (Hynes, 1990). Fibronectin concentration in patient plasma was determined at week 0 and at the end of their treatment by using nephelometry (Behring Nephelometer 100, Hoechst Roussel Canada). The principle of the method is based on the immunoreaction of an antigen with specific antibodies. Proteins present in the sample react with specific antibodies to form insoluble complexes. When light passes through this suspension, a portion of the light is scattered forward by the complexes and focused onto a photodiode by an optical lens system. Quantitation of plasma fibronectin is based on the specific reaction of the fibronectin to be determined with rabbit polyclonal antibodies to human fibronectin. Precipitations are antigen-antibody complexes which show up in solutions as turbidity, scattering incident light and thus generating signals. The quantity of fibronectin is obtained from a reference curve which plots the

relationship between varying concentrations of fibronectin and the measured signal at constant antibody concentration.

Plasma concentrations of fibronectin in both treated groups are presented in Table 4. As previously stated, measurements were made at baseline visit and at the end of study. Duration of treatment for each patient is reported. Plasma levels of fibronectin in patients treated with the 0.2% fibronectin carbomer hydrogel were not statistically different from levels measured in patients receiving the carbomer hydrogel alone.

5.3.4. Anti-fibronectin antibody levels at end of study as measured by ELISA

The possible induction of antibodies to fibronectin during treatment was addressed. A standard curve was previously performed by using polystyrene microtiter plate (NUNC) coated with 100 µL of known concentrations of human IgG from 0.1 to 2.0 µg/100 µL in 50 mM carbonate buffer, pH 9.6 at 4°C overnight.

The determination of anti-fibronectin antibodies in patient plasma was performed as follows. Polystyrene microtiter plate was coated with 100 µL of known concentrations of fibronectin from 0.1 to 2.0 µg/100 µL in 50 mM carbonate buffer, pH 9.6 at 4°C overnight. The plate was washed three times with PBS-Tween buffer and unoccupied binding sites were blocked with 5.0% of BSA in PBS-Tween buffer at 37 °C for 1 hour. After rinsing with PBS-Tween buffer, 100 µL of goat anti-human IgG-horseradish peroxidase conjugated (diluted 2000-fold with 0.5% BSA prepared in PBS-Tween buffer) were added and the plate was incubated 1 hour at 37°C. Excess conjugate was then thoroughly removed by washing, and peroxidase fixed to the wells was detected by addition of ABTS and peroxide substrate diluted in sodium citrate buffer pH 4.6. The reaction was followed by increasing in absorbancy at 410 nm and comparison with a standard human IgG reaction.

Results from studies measuring antibodies to human fibronectin in the plasma of patients from both treatment groups are presented in Table 4. Duration of treatment for each patient is reported. Antibodies to fibronectin were not observed in either group.

Table 4: Fibronectin plasma concentrations and anti-fibronectin antibodies in patient plasma at the beginning and end of study

A. Fibronectin carbomer hydrogel group.

Patient	Baseline visit Plasma level (µg FN/mL)	End of treatment Plasma level (µg FN/mL)	Baseline visit Antibody level (mg/mL)	End of treatment Antibody level (mg/mL)	Patient ended study at week:
GF-101	270	242	0.0	0.0	12
LM-110	204	300	0.0	0.0	24
SD-111	280	166	0.0	0.0	12
HT-113	612	434	0.0	0.0	16
LF-115	414	378	0.0	0.0	12
JC-116	732	402	0.0	0.0	4
MLL-118	316	362	0.0	0.0	12
MG-120	234	266	0.0	0.0	16
FV-124	256	142	0.0	0.0	10
MD-127	396	570	0.0	0.0	8
JGB-131	256	290	0.0	0.0	24
JK-132	206	334	0.0	0.0	12
CR-133	150	142	0.0	0.0	4
LB-140	274	304	0.0	0.0	4
AL-141	258	222	0.0	0.0	12
RL-153	422	436	0.0	0.0	4
TP-155	252	272	0.0	0.0	12
LP-156	202	210	0.0	0.0	14
LPS-157	584	374	0.0	0.0	12
LB-163	152	252	0.0	0.0	10
JK-166	434	422	0.0	0.0	12
LLA-167	174	248	0.0	0.0	6
PD-180	404	286	0.0	0.0	12
Mean	**325**	**307**	**0.0**	**0.0**	**11.5**
Std. Error	**32**	**22**	**0.0**	**0.0**	**2.3**

FN: Fibronectin

B. Carbomer hydrogel alone group.

Patient	Baseline visit Plasma level (µg FN/mL)	End of treatment Plasma level (µg FN/mL)	Baseline visit Antibody level (mg/mL)	End of treatment Antibody level (mg/mL)	Patient ended study at week:
LL-100	178	214	0.0	0.0	12
RV-114	424	428	0.0	0.0	6
BB-117	378	490	0.0	0.0	12
LL-121	154	258	0.0	0.0	2
GA-122	308	360	0.0	0.0	4
JH-125	224	234	0.0	0.0	14
LD-126	288	432	0.0	0.0	6
RM-128	278	260	0.0	0.0	4
GT-129	222	322	0.0	0.0	2
CS-134	290	256	0.0	0.0	2
CAM-135	202	302	0.0	0.0	22
FC-151	390	350	0.0	0.0	4
SC-152	418	366	0.0	0.0	22
MSA-154	392	400	0.0	0.0	4
GR-158	244	268	0.0	0.0	22
CL-159	468	320	0.0	0.0	12
YC-160	260	260	0.0	0.0	6
SG-161	558	454	0.0	0.0	10
SM-164	458	308	0.0	0.0	12
RB-165	212	320	0.0	0.0	2
Mean	**317**	**330**	**0.0**	**0.0**	**9**
Std. Error	**24**	**17**	**0.0**	**0.0**	**2.6**

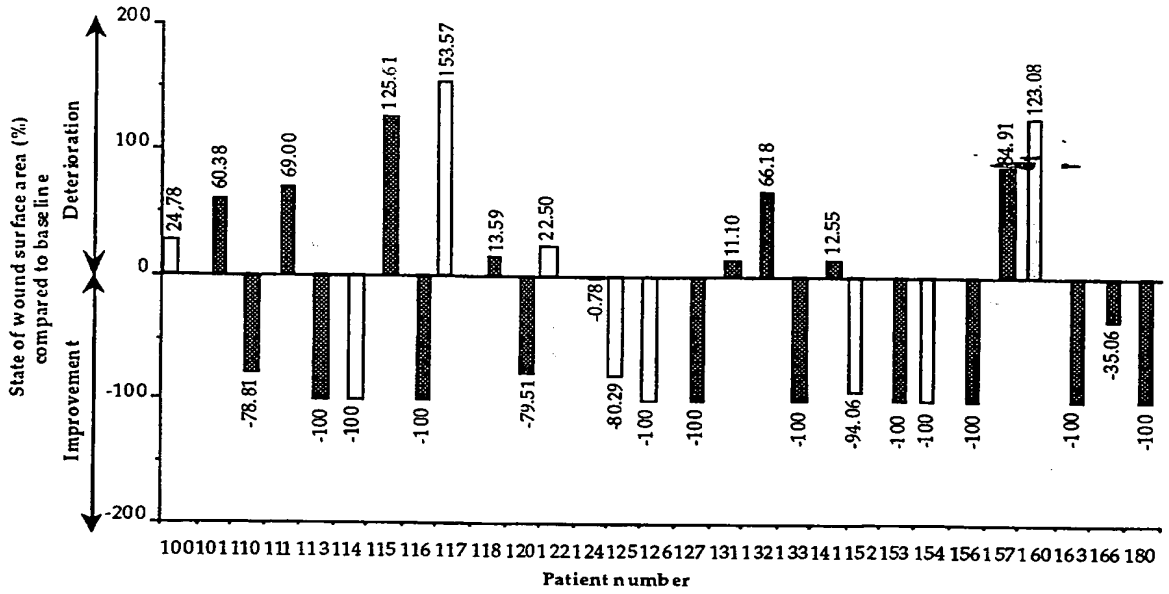
FN: Fibronectin

5.4. Treatment outcome description

Efficacy data analysis, from a statistical standpoint of view, was not reliable given the small sample size. Therefore, outcome of treatment will simply be described. Although 46 patients were randomized in the study, only 29 met the inclusion and exclusion criteria described in the protocol (Annex B). Fifteen (15) additional patients who had a chronic venous leg ulcer were entered in the study in spite of the fact that they did not meet inclusion and exclusion criteria. This was a decision taken in order to increase sample size for safety data acquisition. Two other patients (both in the 0.2% fibronectin hydrogel group) were excluded from treatment outcome description for the following reasons:

> Patient 123 received, theoretically, 8 weeks of treatment, at which time it became obvious to the investigator that very poor compliance had occurred;
>
> Patient 130 received only 4 weeks of treatment. Patient withdrew because of moderate burning sensation and moderate shooting pain at wound site.

The state of wound surface area following treatment in this patient population is presented in Figure 12. The data presented is the treatment outcome in patients with a wound duration time, prior to randomization, of at least 3 months and all patients had failed to respond to at least two other recognized treatment modalities prior to randomization (see protocol for details, Annex B). Duration of treatment was for a maximum of 20 weeks. However, treatment was stopped earlier if complete wound closure occurred before 20 weeks of treatment.



☐ Carbomer Hydrogel alone. ▩ 0.2% Fibronectin Carbomer Hydrogel. All patients had a chronic venous leg ulcer of at least three months duration at study entry and had failed to respond to at least two other treatment modalities. A twice a day application of the hydrogel was performed.

Figure 35: State of wound surface area (%) after a maximum of 20 weeks of treatment in patients with chronic wounds of 3 months minimum duration prior to initiation of treatment-Protocol FN.95.01

Twenty patients were in the 0.2% fibronectin carbomer hydrogel group and 9 patients formed the carbomer hydrogel alone group. Although a comparable proportion of patients had at least an 80% decrease in wound size (>78% healing in Figure 35) in both treatment groups (10/20 for the active treatment group and 5/9 in the control group), in these responding groups, patients who received fibronectin had wounds that were three times more chronic than patients who received carbomer hydrogel alone. Mean duration of wound prior to randomization was 13.4 ± 10.3 months for the fibronectin group and only 4.6 ± 1.2 for the control group (Table 5). In contrast, patients in the non-responding groups were homogenous in terms of both chronicity and wound surface area at study entry (Table 5).

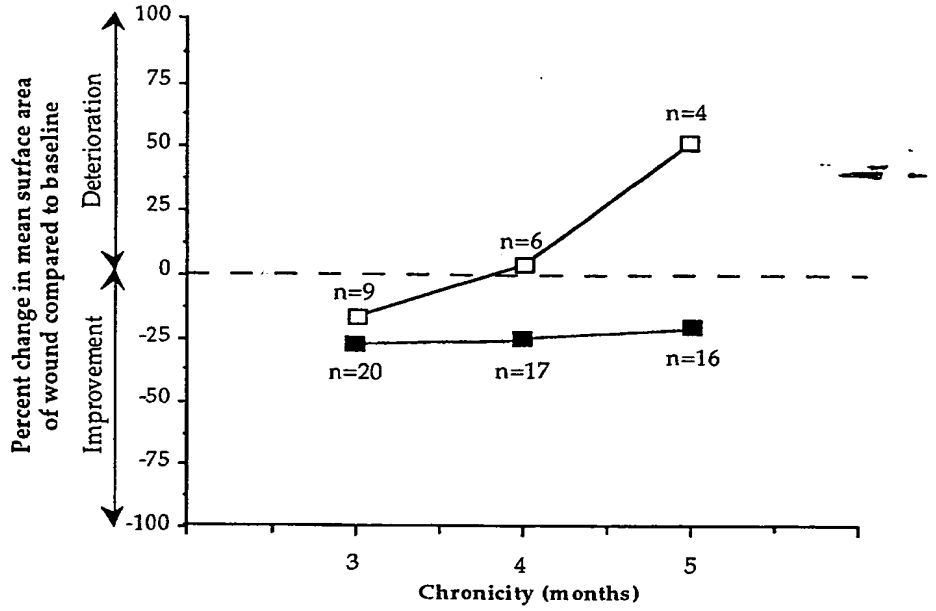
Table 5. State of chronicity and wound surface area at study entry in patients randomized in clinical protocol FN.95.01

Treatment group	> 78% Healing		< 78% Healing or deterioration of wound surface area	
	Months (Mean ± SD)	Surface (cm^2) (Mean ± SD)	Months (Mean ± SD)	Surface (cm^2) (Mean ± SD)
FCH n=20	13.4 ± 10.3 (3 - 37)	2.6 ± 3.7 [0.1 - 13.4]	9.5 ± 4.1 (3 - 17)	3.8 ± 3.2 [0.5 - 11.4]
CH n=9	4.6 ± 1.2 (4 - 7)	3.2 ± 3.4 [0.1 - 9.4]	10.8 ± 7.8 (4 - 24)	2.0 ± 2.1 [0.1 - 5.3]

FCH: 0.2% Fibronectin Carbomer Hydrogel
CH: Carbomer Hydrogel alone
( ): range of months between youngest and oldest wound at study entry
[ ]: range of surface area between smallest and biggest wound at study entry

Since chronicity of wound is likely to be an important factor affecting negatively treatment response, the positive effect seen in this subgroup of patients with very chronic wounds when treated with 0.2% fibronectin carbomer, supports the hypothesis that fibronectin can be beneficial for wound healing. It is also possible that wounds of low chronicity, such as that observed in the control group, are more prone to heal spontaneously, making results of treatment outcome in both groups difficult to compare. Furthermore, by providing a moist and absorbent environment to the wound, hydrogels alone could have some beneficial effect, making the population of patients treated with carbomer hydrogel alone not a true placebo control group.

Moreover, when the data is analysed to examine the % change in mean surface area as a function of chronicity of wound at study entry, it can be observed that as chronicity increases, deterioration of mean wound surface area was much greater for patients treated with the carbomer hydrogel alone group than for those treated with the 0.2% fibronectin carbomer hydrogel (Figure 36). Although the data should be interpreted with great caution in view of the small number of patients at each point, these findings again support the hypothesis of a beneficial effect of fibronectin in wound healing.



☐ Carbomer Hydrogel alone. ▩ 0.2% Fibronectin Carbomer Hydrogel. All patients had a chronic venous leg ulcer of at least three months duration at study entry and had failed to respond to at least two other treatment modalities. A twice a day application of the hydrogel was performed.

Figure 36: Percent change in mean wound surface area compared to baseline in patients with varying degree of wound chronicity at study entry

It must finally be stressed that the amount of fibronectin delivered in the wound (0.4 mg of fibronectin per mm^2 of wound surface area) could have been suboptimal and that a greater proportion of patients might have responded to a higher dose. With the availability of hydrogels containing higher concentrations of fibronectin, significantly more fibronectin will be delivered into the skin wound in future studies. In the above study, only 0.4 µg of fibronectin per mm^2 was delivered into the skin ulcer. With a carbomer hydrogel containing 0.3% fibronectin, as much as 2.5 µg per mm^2 can now be delivered. This amount increases to 3.8 µg with a 0.4% fibronectin carbomer hydrogel and to 5.2 µg for a 0.5% fibronectin carbomer hydrogel. As much as 13 µg can be delivered with a 1% fibronectin carbomer hydrogel (see Figure 5).

REFERENCES

1. Bronaugh, R.L. and R.F. Stewart. 1985. Methods for in vitro percutaneous absorption studies IV: The flow-through diffusion cell. Journal of Pharmaceutical Science. 74: 64-67.

2. Brown, C.D. and J.A. Zitelli. 1993. A review of topical agents for wounds and methods of wounding. Journal of Dermatology Surgery and Oncology. 19: 732-737.

3. Carbopol® water soluble resin service bulletin, GC67 BF Goodrich, Cleveland, 1986.

4. Doran, J.E., A.R. Mansberger and A.C. Reese. 1980. Cold insoluble globulin-enhanced phagocytosis of gelatinized targets by macrophage monolayers: a model system. Journal of the Reticuloendothelial Society. 27 (5): 471-483.

5. Edwards, C.A., M.P.J. Piet and S. Chin et al. 1987. Tri (n-butyl) phosphate/detergent treatement of licensed therapeutic and experimental blood derivatives. Vox Sang. 52: 53-59.

6. Gehrke, S.H. and P.I. Lee. (1990). Hydrogels for drug delivery systems *In* Specialized drug delivery systems manufacturing and production technology. Chapter 8. Volume 41, pp. 333-392, Marcel Dekker, New York.

7. Horowitz, B, R. Bonomo, A.M. Prince, S.N. Chin, B. Brotman and R.W. Shulman. 1992. Solvent / detergent-treated plasma: a virus-inactivated substitute for fresh frozen plasma. Blood. 79 (3): 826-831.

8. Horowitz, B., and M.D.Y. Chang. Preparation of fibronectin for therapeutic administration. *In* Fibronectin, D.F. Mosher ed. San Diego :Academic Press, 1989, 441-455.

9. Hynes, R.O. Fibronectins, Rich A, ed. New-York: Springer-Verlag, 1990, 544 p.

10. Hynes, R.O. Wound healing, inflammation, and fibrosis. In: Fibronectins, Rich A, ed, New-York: Springer-Verlag, 1990: 349-364.

11. Laemmli, U.K. 1994. Denaturing (SDS) discontinuous gel electrophoresis: Laemmli gel method *In* Current Protocols in Molecular Biology, pages 10.2.4 - 10.2.9.

12. Molnar, J., M. Galles, D. Beezhold, M.Z. Lai, C.S-L. Ku and P.J. Van Alten. 1987. Definition of fibronectin-mediated uptake of gelatinized latex by liver slices and macrophage. Biochimica et Biophysica Acta. 930: 326-337.

13. Nishida, T., S. Nakagawa and T. Awata et al. 1982. Rapid preparation of purified autologous fibronectin eyedrops from patient's plasma. Japan Journal of Ophthalmology. 26: 416-424.

14. Phan, T.M., C.S. Foster and S.A. Boruchoff et al. 1987. Topical fibronectin in the treatment of persistent corneal epithelial defects and trophic ulcers. American Journal of Ophthamology. 104: 494-501.

15. Piët, M.P.J., S. Chin, A.M. Prince, B. Brotman, A.M. Cundell and B. Horowitz. 1990.The use of tri(n-butyl) phosphate detergent mixtures to inactivate hepatitis viruses and human immunodeficiency virus in plasma and plasma's subsequent fractionation. Tansfusion. 30 (7): 591-598.

16. Potts, J.R. and I.D. Campbell. 1994. Fibronectin structure and assembly. Current Opinion in Cell Biology. 6: 648-655.

17. Radosevich, M. 1998. Research and development commitments in an integrated plasma collection and plasma fractionation environment. Seminars in thrombosis and hemostasis. 24 (2) 157-161.

18. Regnault, V., C. Rivat, M. Maugras and J.F. Stoltz. 1988. Préparation de fibronectine humaine de haute pureté fonctionellement active. Revue Française de Transfusion et Immuno-hématologie. 31 (1): 19-34.

19. Ruoslahti, E., E.G. Hayman, M. Pierschbacher and E. Engwall. 1982. Fibronectin: purification, immunochemical properties, and biological activities. Methods in Enzymology. 82: 803-831.

20. Wysocki, A., C.R. Baxter and Bergstresser et al. 1988. Topical fibronectin therapy for treatment of a patient with chronic stasis ulcers. Arch Dermatology. 124: 175-177.

Annex A: Composition of different topical formulations studied for fibronectin embodiment

Control

The control consisted of 0.2% fibronectin in phosphate buffered saline pH 7.4.

Lipogel

A lipogel containing (w/w) fibronectin 0.1%, Carbopol 934 P 1% and liposomes 15% from Proliposomes (Pro-lipo 3090 SH®, Lucas Meyer, France) was prepared as follows: a stock solution of fibronectin 1.3 mg/ml (77 ml) was filtered through a 0.22 µm acetate filter. Under sterile conditions, Proliposomes (50 g) were added to fibronectin solution and allowed to dissolve with gentle agitation at 65°C for 15 minutes. The liposomes were isolated by ultracentrifugation at 100,000 g for 60 minutes. The unencapsulated fibronectin in the supernatant was concentrated until desired volume with Centriprep-100® (Amicon, Oakville, Canada). The liposomal preparation (55 g) containing encapsulated and unencapsulated fibronectin and sterile concentrated carbomer base (2.2%) (45 g) previously neutralized with NaOH 3M are mixed into syringes taking care to avoid introducing air bubbles and contamination. This preparation provides a preserved lipogel (100 g) of fibronectin.

Dermabase

A cream formulation containing (w/w) fibronectin 0.1%, sterile cream base (Dermabase®, Borden Ltd, Don Mills, Canada) and chlorocresol 0.1% was prepared as follows: a stock solution of fibronectin 2 mg/mL (50 mL) was filtered through a 0.22 µm acetate filter. Fibronectin solution (50 mL) was then added slowly to a portion (50 g) of the cream base using the low-speed shaft of a stirrer. This provides a preserved cream (100 g) with viscosity of about 120,000 cps.

Carboxymethylcellulose (CMC) hydrogel

Carboxymethylcellulose (CMC) hydrogel was prepared. Preferred grade is GPR® (BDH Laboratories, Ville St-Laurent, Canada) at concentrations ranging from 2 to 4%

(w/w) for CMC hydrogel. A hydrogel formulation containing (w/w) fibronectin 0.1%, CMC 3% and parabens was prepared as follows: methylparaben (0.05 g) and propylparaben (0.02 g) were dissolved in warm deionized water (94 mL). CMC powder was sterilized by using a dry-heat sterilization process. CMC (6 g) was then dispersed in this solution and allowed to be mixed with a paddle type stirrer for about 3 hours. This provides a sterile concentrated hydrogel base (6% w/w). A stock solution of fibronectin 2 mg/mL (50 mL) was filtered through a 0.22 µm acetate filter. Fibronectin solution (50 mL) was then slowly added to a portion (50 g) of this concentrated base using the low-speed shaft of the stirrer. This provides a preserved gel (100 g) with viscosity of about 400,000 cps.

Carbomer Hydrogel containing 10% glycerol (Carbogly)

The carbomer hydrogel containing 10 % glycerol is prepared as follows: chlorocresol (1 g) is dissolved in warm deionized water (95 mL). Carbomer (3.75 g) is then dispersed into this solution and allowed to be mixed with a paddle type stirrer for about 3 hours. This dispersion is then autoclaved to provide a sterile concentrated hydrogel base (3.75% w/w). A stock solution of fibronectin ranging from 2.2 to 10 mg/mL (80 mL) and containing 10 g of glycerol is filtered through a 0.22 µm acetate filter. A polymerization promoter, sodium hydroxide, is then added to the fibronectin solution containing glycerol in an amount that will neutralize a 10 g portion of the carbomer 3.75% dispersion, that is 1250 µL of NaOH 3M. The stock solution of fibronectin containing glycerol and a 10 g portion of the carbomer dispersion are mixed into syringes taking care to avoid introducing air bubbles and contamination. This preparation provides a clear, preserved hydrogel (100 g) of fibronectin containing 10% of glycerol, free from microorganisms and with viscosity of about 570 000 cps.

Schering base

A cream formulation containing (w/w) fibronectin 0.1%, sterile cream base (Schering® base, Schering, Pointe-Claire, Canada) and chlorocresol 0.1% was prepared as follows: a stock solution of fibronectin 2 mg/mL (50 mL) was filtered through a 0.22 µm acetate filter. Fibronectin solution (50 mL) was then added slowly to a portion (50 g) of the cream base using the low-speed shaft of a stirrer. This provides a preserved cream (100 g) with viscosity of about 80,000 cps.

Poloxamer

Polyoxyethylene-polyoxypropylene block copolymer (poloxamer) gels (Pluronic®, BASF Wyandotte) were prepared. Preferred grade of poloxamer is Pluronic® F-127 at concentrations ranging from 18 to 25% (w/w). Concentrated aqueous solution (20 to 30%) have been reported to show a dramatic increase in viscosity when heated from 4°C to body temperature. Furthermore, if the ionic strength of the solution is increased, this brings the viscosity to increase more rapidly with temperature. Several grades are available but the F-127 grade is the least toxic and the one with which gelation can occur at lower concentrations. Hydrogels of poloxamer prepared in this drug substance are low viscosity solutions at 4°C and gelify rapidly when they are warmed to body temperature.

A poloxamer hydrogel containing (w/w) fibronectin 0.2% and Pluronic® F-127 20% was prepared as follows: a stock solution of fibronectin 2.2 mg/mL (80 mL) was filtered through a 0.22 µm acetate filter. Pluronic® F-127 (20 g) was added to 80 mL of the fibronectin solution and allowed to dissolve without agitation at 4°C for about 3 days. This provides a solution (100 g) which is very liquid like. Gelation instantaneously occurs when the solution comes into contact with the wound. A sterilizing filtration process performed at 4°C could also be applied to the final solution if the sterile poloxamer powder can not be obtained...Viscosity varies from not detectable values at 4°C to 450,000 cps at room temperature.

Hydroxypropylcellulose (HPC) hydrogel

Hydroxypropylcellulose hydrogel was prepared. Preferred grade is Klucel-HF® (Aqualon, Houston, Texas) at concentrations ranging from 2 to 4% (w/w) for HPC hydrogel. A hydrogel formulation containing (w/w) fibronectin 0.1%, HPC 3% and parabens was prepared as follows: methylparaben (0.05 g) and propylparaben (0.02 g) were dissolved in warm deionized water (94 mL). HPC powder was sterilized using a dry-heat sterilization process. HPC (6 g) was then dispersed in this solution and allowed to be mixed with a paddle type stirrer for about 3 hours. This provides a sterile concentrated hydrogel base (6% w/w). A stock solution of fibronectin

2 mg/mL (50 mL) was filtered through a 0.22 µm acetate filter. Fibronectin solution (50 mL) was then slowly added to a portion (50 g) of this concentrated base using the low-speed shaft of the stirrer. This provides a preserved hydrogel (100 g) with viscosity of about 150,000 cps.

Annex B: Clinical protocol FN-95-01

A randomized, double blind, placebo controlled trial on efficacy and safety of topically applied homologous human plasma fibronectin in the treatment of chronic venous leg ulcers. (FN-95-01)

February 1995

TABLE OF CONTENTS

I- ABSTRACT

This is a randomized, double blind, placebo controlled trial on efficacy and safety of a topical dosage form containing fibronectin in the treatment of chronic venous leg ulcers. Fibronectin or placebo will be applied twice a day on a chronic venous ulcer present for at least three months and which has been shown to be resistant to at least two conventional treatments. Patients will be assessed every other week until complete reepithelialization or withdrawal from the study with particular attention to the surface of the ulcer and adverse effects.

II- INTRODUCTION

The chronic venous leg ulcer is a common disorder. In the United States, there are about 500,000 to 600,000 individuals affected by chronic ulcers of lower limbs. It is estimated that about 80 to 90% of these cases are caused by deep venous insufficiency. Annual costs involved in the treatment of this disorder approximate one billion dollars. Moreover, this problem would be responsible for the loss of about two millions days-work per year. The treatment of the cause of the ulcer is not always possible or sufficient to promote wound healing. Several treatments are available including topical antibiotics, hydrocolloid dressings, and as an investigational therapy, keratinocyte culture. Although these treatments might be useful, they are only adjuvant.

A- Fibronectin as a wound healing promoter

Fibronectin is a glycoprotein that normally plays a major role in the healing of skin wounds. In the chronic venous ulcer, fibronectin has been shown to be either absent or present in only limited amount. Fibronectin is normally found in plasma at a concentration of about 300 µg/ml. It plays a major role in all phases of the normal wound healing process. These phases are: synthesis of a temporary matrix, neoangiogenesis associated with the synthesis of the granulation tissue, reepithelialization and, scar remodeling. During temporary matrix synthesis, there is accumulation of fibrinogen and fibronectin. At this stage, fibronectin promotes fibroblasts migration into the wound and debris phagocytosis by the monocyte. Then the angiogenesis takes place with endothelial cell migration. It has been clearly shown that fibronectin exerts chemotactic activity on endothelial cells. The epithelium migrates on the basal membrane which ultimately leads to the reepithelialization of the wound. Unless the skin has been wounded, there is normally no fibronectin in the basal membrane. When this occurs, fibronectin appears and promotes epithelial cell migration onto the basal membrane. When the reepithelialization process is ended, fibronectin disappears from the basal membrane. The healing process ends with the remodeling phase. It has been shown that the remodeling tissue contains large amounts of fibronectin which promote its synthesis by exerting a chemotactic effect and by strengtening the link between fibroblasts and extracellular matrix components such as collagen. It is obvious that fibronectin plays a major role in all stages of the normal wound healing process. As shown by immunofluorescence techniques and in contrast

with ulcers that heal normally and where fibronectin can be found in large amounts, there is no fibronectin in tissues of chronic venous ulcers. Moreover, animal studies have shown that the application of exogenous fibronectin on a skin incision accelerates the wound healing process by halving the time required to achieve wound closure. So, it is possible that the topical application of exogenous fibronectin might accelerate and promote the healing of chronic wounds in human. This hypothesis should be studied.

B- Preliminary studies

During the last three years, we have performed four pilot studies in order to assess the therapeutic potential of different topical formulations containing human plasma fibronectin. In these studies, human plasma fibronectin isolated from patients' blood (autologous fibronectin) was used. The chronic venous leg ulcer must have been present for at least three months.

In a first study, 7 patients were treated with a solution containing 0.1% (w/v) fibronectin. Patients were instructed to flood their wound twice a day with this solution for three months. In 5 of 7 patients studied, there was a spectacular reduction in the dimension of the ulcer. The dimension of the ulcer was determined by computerized analysis of the integrated surface of the ulcer and to a lesser extent by photographic analysis. In all cases, a reduction of the surface of the ulcer of at least 75% was noted during the first three months of treatment.

Subsequent studies were undertaken in order to assess the efficacy of a semi-solid topical vehicle as an alternative to the fibronectin solution. Hypothesis was that if the contact time of the protein with the wound could be enhanced, a more rapid decrease in the healing time could theoretically be observed. The effectiveness seen with such a formulation should be at least comparable to that seen with the solution. Furthermore, the gel formulation should reduce significant losses of fibronectin caused by the flow of the solution on the lower limbs.

A second study including six patients was performed with a gel containing 0.1% (w/w) fibronectin encapsulated in liposomes. This gel is referred as gel #1. Patients were instructed to apply the gel twice a day on the ulcer. The dose was proportional to the wound size. The mean treatment duration was three months. None of these patients presented a significant improvement of his ulcer.

In a third study, 11 patients were treated with a 0.2% (w/w) gel of fibronectin (gel #2). Patients applied this gel twice a day for three months. In 3 of 11 patients (27%), a reduction of more than 50% of the integrated surface of the ulcer was noted. The relative ineffectiveness of this gel might be explained, at least in part, by the results of the absorption studies. These studies have shown that the dermal absorption of fibronectin was much more important with the fibronectin solution compared with the gel. However, the application of a topical solution is unsuitable for clinical utilization and often results in significant losses of fibronectin outside the wound.

In the meantime, a model was developed in order to assess delivery characteristics of different topical vehicles. This model was an adaptation of the diffusion cell system on which a deepithelialized human skin specimen was placed. The deepithelialized skin reproduces the pathological condition of the skin wound where the epidermal layer is absent. A radio-labeled sample of the fibronectin gel is placed on the skin and radioactivity counts are regularly performed in order to determine the amount of labeled fibronectin found in the dermis at a given time. Permeation studies performed with this model have shown a correlation between the degree of improvement in clinical pilot studies and delivery characteristics of the studied vehicles. In these studies gel #1 showed very poor delivery capabilities and gel #2 showed good delivery capabilities. Subsequently, this model has led to the development of an optimal topical vehicle (gel #3) which promotes the dermal absorption of large amounts of fibronectin.

Eight patients were recently treated with this gel (gel #3) in order to determine the therapeutic potential of this topical formulation. Results are promising since a reduction of more than 50% of the wound size has been noted in 50% of patients after three months of treatment, including two wound closure cases after an eight-week course of treatment. It should be remembered that all these patients had chronic venous leg ulcers that were known to be highly resistant to the conventional therapy.

Studies assessing macro-structure integrity and biological activity of fibronectin in this gel are in progress. Preliminary data indicate that fibronectin is stable for at least 14 months in this gel when refrigerated.

It should be noted that these studies were not placebo-controlled. Results obtained from these pilot studies have to be validated by a randomized, double blind, placebo-controlled

study. A topical gel promoting the release of large amounts of fibronectin to the dermis has been developed and will be assessed in this study.

III- OBJECTIVE

The objective of the study is to assess the efficacy and safety of a topical dosage form containing homologous human plasma fibronectin versus a placebo in the treatment of chronic venous leg ulcer in patients with venous insufficiency.

IV- TOPICAL GEL OF FIBRONECTIN

A- Purification of fibronectin

In this study, fibronectin will be isolated from healthy donors' plasma by chromatography using gelatin Sepharose 4B. About 4 plasmapheresis will be performed in each donor at the rate of one plasmapheresis weekly. Donors' blood will be screened in order to eliminate risks of infectious disease transmission. These tests will be performed two times on donors, that is during the week before the first plasmapheresis and six months after the last plasmapheresis. In the meantime, donors' plasma will be frozen (-20°C) and kept in quarantine. Blood specimens will be analyzed by the Canadian Red Cross Society in order to detect a potential contamination by the hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), human T-cell lymphotrophic virus (HTLV), syphilis, cytomegalovirus (CMV) and, atypical antibodies. Laboratory tests used are micro-hemagglutination (syphilis), enzymatic-immunologic assay - EIA - (HIV and HBV), ELISA (HTLV and HCV), passive agglutination on latex (CMV) and agglutination on red blood cells (atypical antibodies). In addition, a known virus inactivation process (solvent/detergent method) will be performed on all plasma donations.

B- Description of the topical dosage form

The topical dosage form will consist of a 0.2% by weight gel of fibronectin. The placebo gel will contain no fibronectin. It has been shown that fibronectin does not alter physical properties and the appearance of the gel. Therefore, the addition of a control protein in the gel is not required.

V- STUDY DESIGN

A- Description

This is a multicentre, randomized, double blind, placebo-controlled study. Each subject will be assessed by the same dermatologist and/or the same nurse at the following visits: two weeks before the randomization (observation phase), every other week during the active treatment phase and one month and three months after treatment completion. A dosage of anti-fibronectin antibodies is also scheduled one year after treatment completion. If the subject meets inclusion and exclusion criteria, he will be randomized and will be treated in a double blind manner either with the fibronectin gel or the placebo gel. The following steps of the study protocol are shown in appendix 1.

B- Duration

The minimal duration of this study is 14 weeks, including a two-week observation phase and a 12-week treatment phase. A 12-week prolongation is also planned for patients who will present at visit *week 12* with an improvement $\geq 30\%$ of the surface of their ulcer compared to their initial surface (as measured at visit *week 0*). Follow-up visits are also scheduled one and three months and one year after treatment completion.

C- Number of subjects

Sixty (60) subjects, men or women, between 18 and 85 years, presenting with venous insufficiency and a chronic venous leg ulcer will be enrolled. These subjects will have to meet inclusion and exclusion criteria listed in section V (Method and assessment) to be randomized.

VI- METHOD AND ASSESSMENT

A- METHOD

Subjects will be recruited in participating centers. Each patient will be assessed by the same dermatologist and/or the same nurse for all the study duration. The patient will have to meet inclusion and exclusion criteria to enter the treatment phase (fibronectin *vs* placebo). The patient will be assessed once before double blind treatment initiation (visit *week -2*), at the beginning of the treatment phase (visit *week 0*), every other week during the active treatment phase and one month and three months after treatment completion. Assessments must be performed every 14 days or within five (5) days of this date. A dosage of anti-fibronectin antibodies is also scheduled one year after treatment completion in order to verify if these antibodies appeared in the patient's blood.

During the observation phase (prior to randomization), the patient will stop any active treatment for his ulcer (except the local compression) and will apply the placebo gel. During the next 12 weeks of treatment, the patient will apply either the fibronectin gel or the placebo gel. At the end of this 12-week period, if the improvement of the ulcer is ≥ 30% compared to the initial surface (as measured at *week 0*), the patient will continue with the same gel; if the improvement is < 30%, he will be withdrawn from the study. Follow-up visits are scheduled one month and three months after study completion for all patients.

The patient will clean his ulcer with boiled water, salted or not, using a sterile gauze. Then he will apply enough gel to cover the wound surface with a thin layer (about 0.5 mm) of gel. The amount of gel required will be determined from the dimension of the ulcer, that is its surface, and will be within the range of 0.05 cm^3 to 1 cm^3. In order to control the amount of gel applied, it will be supplied to patients in unidose syringes. A dressing such as Telfa and a dry gauze associated with a bandage, if necessary, will cover the gel. This step will be repeated twice a day, in the morning and the evening, during both the observation phase and the treatment phase.

The group using the fibronectin gel will include as many patients as the group that will receive the placebo gel (see section X: Statistical issues). Each patient will be identified by a numerical code that relates to the fibronectin gel or the placebo gel. This code will

be sealed before the beginning of the study and will be kept secret until the end of the study. However, should an emergency occur, the code will be disclosed to the investigator. The patient will have to comply with some activity criteria as mentioned under the section "CO-INTERVENTIONS".

1- Inclusion criteria:

— Patient with a chronic venous ulcer of the lower limbs.

— Ulcer present for at least three (3) to sixty (60) months before the visit *week -2*.

— Ulcer lesser than 20 cm^2 of integrated surface.

— Patient must be willing to cooperate and must have signed the consent form.

— Ambulatory patient between 18 and 85 years.

— Patient who has utilized two (2) different conventional treatments for the current episode. Examples of conventional treatments are: local compression to the affected limb, hydrocolloid dressings, dressings with normal saline, topical antibiotics, Sofra-tulle, Adaptic, Pace's technique, Opsite, Tegaderm, Dakin, whirlpool, failure of a skin graft trial.

2- Exclusion criteria:

— Medical, surgical and/or dermatological disorders that might interfere with the course of the ulcer:

- vasculitis
- arterial insufficiency (ankle/arm index less than 70% or 70 mm Hg (in absolute value))
- clinical hypothyroidism
- medication that might interfere with the course of the ulcer: chemotherapy agent, prednisone (more than 7.5 mg/day), topical medication for the ulcer (different from the study drug)
- significant heart failure, renal failure, liver failure
- significant alcoholism
- significant drug addiction
- significant biological disorder
- vascular surgery during the study
- sclerotherapy during the study

- all medical condition likely to interfere with the study as judged by the physician-investigator
- positive serum pregnancy test in a woman of childbearing potential

— Ulcer with an exposed bone and/or tendon and/or muscle

B- ASSESSMENT

1- Assessment at visit *week -2*

— Inclusion and exclusion criteria.
— Signature of the consent form.
— Demography (age, sex, race, date of birth).
— Ulcer history with previous treatments and adverse effects.
— Medical history: previous medical and surgical problems with duration; allergy, cigarette smoking, alcohol consumption.
— Concurrent medication.
— Adverse effects.
— Physical examination:

- Weight, height, vital signs: blood pressure, heart rate, respiratory rate, oral temperature (°C)
- Physical examination: general appearance, eyes, ears, nose, throat, thyroid, heart, lungs, abdomen, musculoskeletal system, uro-genital system, skin.
- Examination of the arterial vasculature: femoral, posterior tibial, and pedal pulse with gradation from 0 to 3 +, presence of livedo, cyanosis, capillary filling time, ankle/arm index.
- Measurement of the lower limbs.
- Examination of the lower limbs: edema, induration, hemosiderosis, Milian's white atrophy, purpura, eczema, dry skin.
- Examination of the ulcer with description: ulcer location, presence of necrotic tissue, presence of granulation tissue, presence of fibrinous tissue, crusted surface, sclerosed bottom, redness around the ulcer, swelling around the ulcer, pain, pain characteristics, pain severity, wound discharge, discharge characteristics.
- Measurement of the integrated surface of the ulcer.
- Ulcer location (drawing in the case report form).

— Clinical laboratory tests:

- CBC, erythrocyte sedimentation rate, urea, creatinine, ALT, AST, LDH, TSH, β-HCG (if applicable), fasting blood glucose, albumin, urinalysis with microscopic examination.
- Serum fibronectin
- Anti-fibronectin antibodies

— Placebo gel delivery.
— Photographs of the ulcer.
— Comments.
— Signatures.

2- Assessment at visit *week 0*

— Inclusion and exclusion criteria.
— Randomization.
— Concurrent medication.
— Adverse effects.
— Physical examination:

- Vital signs: blood pressure, heart rate, respiratory rate, oral temperature (°C).
- Measurement of the lower limbs.
- Examination of the lower limbs: (as defined above).
- Examination of the ulcer: (as defined above).
- Measurement of the integrated surface of the ulcer.

— Clinical laboratory tests:

- Microbiological culture of the wound bed.
- Serum fibronectin.

— Delivery of the notebook (to record daily activities) and the instructions sheet to the patient.
— Gel delivery (double blind treatment).
— Compliance assessment.
— Photographs of the ulcer.
— Comments.
— Signatures.

3- **Assessment every other week**

— Concurrent medication.
— Adverse effects.
— Physical examination:
  - Vital signs: blood pressure, heart rate, respiratory rate, oral temperature (°C).
  - Weight at visits *week 6, 12* and *20*.
  - Measurement of the lower limbs.
  - Examination of the lower limbs at visits *week 6, 12* and *20*: (as defined above).
  - Examination of the ulcer: (as defined above).
  - Measurement of the integrated surface of the ulcer.
— Ulcer improvement assessment (%) at visit *week 12*.
— Physician and patient global assessment.
— Clinical laboratory tests:
  - Serum fibronectin every 4 weeks (from *week 0*).
  - Anti-fibronectin antibodies at visit *week 12*.
— Photographs of the ulcer at visit *week 12*.
— Notebook return and delivery.
— Syringes return and gel delivery.
— Compliance assessment.
— Comments.
— Signatures.

4- **Assessment at visit *final* (premature discontinuation, complete reepithelialization or study completion)**

— Physical examination:
  - Vital signs: blood pressure, heart rate, respiratory rate, oral temperature (°C).
  - Measurement of the lower limbs.
  - Examination of the lower limbs: (as defined above).
  - Examination of the ulcer: (as defined above).
  - Measurement of the integrated surface of the ulcer.

— Clinical laboratory tests:

- CBC, erythrocyte sedimentation rate, urea, creatinine, ALT, AST, LDH, blood glucose, albumin, urinalysis with microscopic examination.
- Microbiological culture of the wound bed (if applicable).
- Serum fibronectin.

— Compliance assessment.
— Photographs of the ulcer.
— Reasons for premature discontinuation (if applicable) OR conclusion.
— Comments.
— Signatures.

5- Assessment at visits *one month* and *three months* post treatment

— Concurrent medication.
— Adverse effects.
— Physical examination:

- Weight, vital signs: blood pressure, heart rate, respiratory rate, oral temperature (°C).
- Measurement of the lower limbs.
- Examination of the lower limbs: (as defined above).
- Examination of the ulcer: (as defined above).
- Measurement of the integrated surface of the ulcer (if applicable).

— Physician and patient global assessment.
— Clinical laboratory tests:

- Serum fibronectin.
- Anti-fibronectin antibodies at visit *3 months*.

— Photographs of the ulcer.
— Comments.
— Signatures.

6- Assessment at visit *one year* post treatment

— Clinical laboratory test:

- Anti-fibronectin antibodies

C- COMPLIANCE

The patient will be considered compliant to treatment if at least 80% of syringes supplied have been used.

VII- PREMATURE DISCONTINUATION

A- Withdrawal conditions

The participation of a patient in the study may be prematurely discontinued if:

— the patient chooses to withdraw from the study.
— the patient is hospitalized during the study.
— the patient becomes pregnant during the study.
— the investigator recommends patient withdrawal from the study

When such a situation occurs, the investigator must:

1- Fill the section visit *final* in the case report form,
2- Assess the availability of a substitution treatment for the ulcer.

The investigator must record the reasons for premature discontinuation in the case report form. The physical examination and clinical tests scheduled at visit *final* must be done when the patient is withdrawn from the study or as soon as possible after this date.

B- Replacement of subjects

Subjects that will withdraw from the study or who will be withdrawn before completion of a 12-week course of treatment will be replaced. This procedure does not apply if withdrawal is due to an adverse effect.

VIII- CONTRAINDICATIONS, PRECAUTIONS AND ADVERSE EFFECTS

A- Contraindications and precautions

Application of the gel is contraindicated in patients with a known allergy to the gel components (carbomer and chlorocresol).

The patient should not apply any ointments, creams, dressings or any other treatments to his ulcer during the study.

Blood levels of fibronectin will be determined at the visit *week -2*, every four weeks during the active treatment phase and one month and three months after treatment completion. The specimen will be analyzed by nephelometry. This blood test is performed in order to make sure that topical treatment with fibronectin does not result in abnormal increases in plasma levels of fibronectin.

An anti-fibronectin antibody dosage will be performed four times in all patients. These dosages are scheduled at visit *week -2*, at visit *week 12*, and three months and one year after study completion.

B- Adverse effects

Reactions to the gel components, containing fibronectin or not, such as itching, redness, localized edema and burn sensation may occur.

Blood punctures may be painful and bruise may appear at injection sites. Local infection may also rarely occur.

Any adverse effect associated with or assumed to be associated with the study drug (fibronectin vs placebo) must be recorded on the adverse effects notification sheet in the case report form. Adverse effects such as reactions to the study drug, concurrent illness exacerbation during the study, emergence of new symptoms or of a new illness during the study must be recorded.

In order to find the origin of the adverse effect and to determine if it should be classified as a serious adverse effect (see section C), the investigator should obtain all relevant information on the adverse event. The follow-up of a serious adverse effect should be performed every other week until symptom resolution or until the symptom(s) is judged acceptable by the investigator.

C. Serious adverse effects

Any serious adverse effect (as defined hereafter) associated with or assumed to be associated with the study drug must be immediately communicated by telephone to the principal investigator: Dr André Beaulieu (418) 654-2772.

A serious adverse effect refers to any event that:

- is fatal
- is life threatening
- is permanently disabling
- requires, or prolongs, hospitalization
- is a congenital anomaly
- is a cancer
- is an overdose

Any adverse effect considered serious by the investigator must also be communicated immediately.

A serious adverse effect or a death occuring during the study or within the 30 days following the visit *final* must be immediately communicated to the principal investigator, whatever the cause may be.

For any serious adverse effect, the investigator will be required to provide all relevant information to the principal investigator in addition to information registered in the case report form. Data communicated by telephone should be accurate and should reflect those mentioned in the case report form. A copy of forms to complete in case of serious adverse effect is available in appendices III and IV. In case of a death, a

necropsy summary, if available, must be sent to the principal investigator as soon as possible.

IX- CO-INTERVENTIONS

The patient will have to raise the foot of the bed to form an angle of about 20 degrees, that is to say that he will place either a two-inch block under the foot of the bed or two pillows between the base and the mattress of the bed.

If the patient remains seated for more than 30 minutes, he will have to raise his legs. If the patient remains in a fixed standing position, he will be instructed to walk every 30 minutes.

The patient will not have to use external compression such as Velpeau bandage, support stockings or other compression devices unless he uses it regularly.

In order to complete data, the patient will have to record daily activities in a notebook and to return it to his physician at each visit from visit *week 0* to visit *final*.

X - STATISTICAL ISSUES

Based on our previous pilot study, percentage of patients showing a reduction of at least 50% of their ulcer in three months should be at least 75%. Such improvement in patients using the placebo gel should be about 10 to 15%. By fixing the alpha error at 0.05 and the beta error at 80%, 48 patients (24/group) will be required to demonstrate such a difference between the groups. This number will be rounded to 30 patients per group in order to take into account patients that will not be compliant to treatment or who will be withdrawn from the study.

XI- REFERENCES

— Harahap M. Leg ulcers. Clin Dermatol 1990; 8(34).

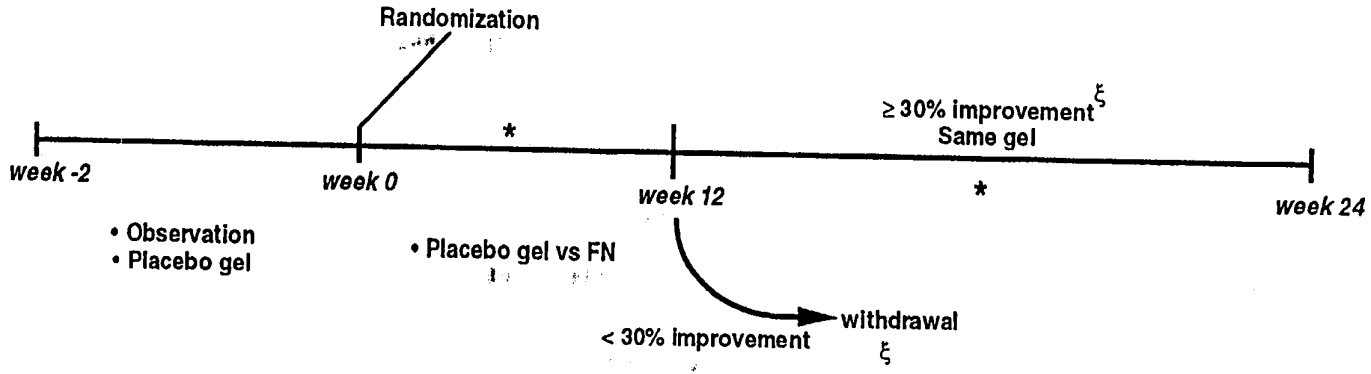
— Dover J, Philips T. Leg ulcers. J Am Acad Dermatol 1991; 25: 965-87.

— Eaglestein WH. Wound healing. Clin Dermatol 1984; 2(3).

— Mulder GD. Clinical protocols for studying wound healing. Clin Materials 1991; 8:251-5.

APPENDIX 1

STUDY DESIGN



FN	=	homologous human plasma fibronectin
*	=	visits every other week
ξ	=	improvement from *week 0*

APPENDIX FOLLOW-UP

VISITS	Week -2	Week 0	Every 2 weeks	Every 4 weeks	Week 6-12-20	Final	1 and 3 months post Tx	1 year post Tx
CONSENT FORM	•							
INCLUSION/EXCLUSION CRITERIA	•							
RANDOMIZATION		•						
ULCER HISTORY	•							
DEMOGRAPHY	•							
MEDICAL HISTORY, HEIGHT	•							
VITAL SIGNS	•	•	•			•	•	
WEIGHT	•				•		•	
CONCURRENT MEDICATION	•	•	•				•	
ADVERSE EFFECTS	•	•	•				•	
PHYSICAL EXAMINATION	•							
EXAMINATION OF THE ARTERIAL VASCULATURE	•							
MEASUREMENT OF THE LOWER LIMBS	•	•	•			•	•	
EXAMINATION OF THE LOWER LIMBS	•	•			•	•	•	
EXAMINATION OF THE ULCER	•	•	•			•	•	
MEASUREMENT OF THE ULCER	•	•	•			•	•	
LOCALISATION OF THE ULCER (DRAWING)	•							
BLOOD TESTS	•					•		
URINALYSIS	•					•		
MICROBIOLOGICAL CULTURE OF THE WOUND BED		•				•		
SERUM FIBRONECTIN	•	•		•		•	•	
ANTI-FIBRONECTIN ANTIBODIES	•				• (week 12)		• (vis. 3 months)	•
PHOTOGRAPHIES OF THE ULCER	•	•			• (week 12)	•	•	
NOTEBOOK (TO RECORD DAILY ACTIVITIES)		•	•					
PHYSICIAN GLOBAL ASSESSMENT			•				•	
PATIENT GLOBAL ASSESSMENT			•				•	
COMPLIANCE		•	•			•		
SIGNATURES	•	•	•			•	•	

We claim:

1. A solid dressing comprising an effective level of a wound healing promoter.

2. A solid dressing according to claim 1, wherein the wound healing promoter is fibronectin.

3. A solid dressing according to claim 1, which contains at least 0.05 to 1.0% of fibronectin by weight.